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Transcriptional control of PIN auxin efflux carriers by cytokinin

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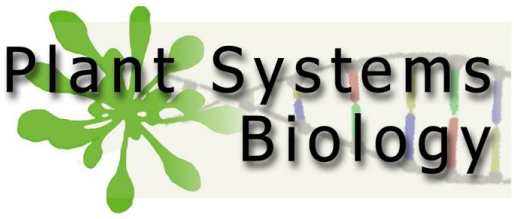
VIB


Plant Systems
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“Meet the true lords of life, the plants. Life could get along without animals and without fungi. But abolish the plants, and life would rapidly cease.”

- Richard Dawkins

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Frequently used abbreviations

ACC: 1-aminocyclopropane-1-carboxylate

AD: Activation domain

AP2: APETALA2

AHK: ARABIDOPSIS HISTIDINE PROTEIN KINASE

ARF: AUXIN RESPONSE FACTOR

ARR: ARABIDOPSIS RESPONSE REGULATOR

AVG: Aminoethoxyvinylglycine

AuxRE: auxin responsive element

AUX1: AUXIN RESISTANT1

Aux/IAA: auxin/indole-3-acetic acid; auxin-responsive protein / indoleacetic acid-induced protein

BA: 6-benzylaminopurine

ChIP: Chromatin immunoprecipitation

CK: cytokinin

CRF: CYTOKININ RESPONSE FACTOR

CKX: oxidases/dehydrogenase

Col-0: Columbia-0 ecotype

ERF: ETHYLENE-RESPONSE FACTOR

FC: founder cell

GFP: Green Fluorescent Protein

GUS: *β*-glucuronidase

IAA: indole-3-acetic acid

IPT: isopentenyltransferase

LAX: LIKE-AUX1

LOG: LONELY GUY

LR: lateral roots

LRD: lateral root density

LRI: lateral root initiation

LRP: lateral root primordium

MS: Murashige and Skoog basal salt mixture

PAT: polar auxin transport

PI: propidium iodide

QC: quiescent canter

Q-RT-PCR: quantitative reverse transcriptase polymerase chain reaction

RAM: Root apical meristem

RM: Root meristem

RFP: red fluorescent protein

SAM: Shoot apical meristem

TF: transcription factor

TIR1: TRANSPORT INHIBITOR RESPONSE1

WT: wild type

YUC: YUCCA

Y1H: Yeast-one-hybrid

2,4-D: 2,4-dichlorophenoxy acetic acid

Scope

The plant body exhibits an amazing flexibility that enables it to cope with different environmental conditions and developmental signals. This plasticity is achieved by a tight balance between maintenance of the stem cell identity and at the same time promotion of the daughter cell proliferation to differentiate into specific cell types and, thus; form new organs during the entire plant life. Stem cells are established at the correct position already at early stages of embryonic development and serve as reference for the future root and shoot meristems. The two plant hormones -auxin and cytokinin- are involved in the regulation of these processes. Early experiments have demonstrated the crucial roles played by both hormones in cell proliferation and new organ generation. It is striking that the cell fate decision in specific tissues is determined by the ratio between auxin and cytokinin, which is translated by the tissue in cell proliferation maintenance or cell differentiation stimulation to create new organs, such as shoots or roots.

Increasing information about the biosynthesis, transport, and signaling of auxin and cytokinin together with improved genetic and molecular tools over the past years have helped to unravel multiple aspects of their interaction at the transcriptional and posttranslational levels. However, when the extensive functional redundancy among gene family members and multiple regulatory feedback circuits in both hormone pathways are taken into account, the crosstalk between these two hormones is still poorly understood. Several studies have proposed that cytokinin and auxin mutually regulate their signaling pathways or their metabolisms through certain integrating factors, but, thus far, only a few of these components that are the crosstalk backbone between these two hormones have been demonstrated to determine a specific developmental outcome.

The aim of this study was to gain insight into the cytokinin-mediated regulation of auxin transport and distribution at the transcriptional level and to identify new components that integrate auxin transport and cytokinin signaling. The Yeast-One Hybrid assay was used as a molecular tool to detect new protein-DNA interactions. We performed a reverse-genetics screen to isolate candidate transcription factors that can bind to specific cytokinin response factors located in the promoters of the *PIN-FORMED1 (PIN1)* and *PIN7* auxin efflux carriers.

After the found specific protein-DNA interactions were confirmed *in planta*, a detailed phenotypic analysis of gain- and loss-of-function mutants of candidate interactors hinted at the involvement of these genes in fine-tuning the activity of the polar auxin transport machinery in response to cytokinin during plant growth and development.

Chapter 1.

Introduction

Author contributions: M.S wrote the chapter, E.B saw and commented on the manuscript.

Plant hormones shaping the plant body

Plants and animals have diverged approximately 1.5 billion years ago[1]. Although both share the same genetic heritage of their common unicellular eukaryotic ancestor, their multicellular organization has evolved independently. Despite numerous similarities between plants and animals, most of the contrasts in their development arise from two basic features of plants. First, the ability to produce energy from light, and not from ingesting other organisms as animal do, shapes a very distinct body plan. The second major difference between plant and animal cells is the presence of a rigid cell wall, characteristically absent in the animal kingdom, preventing plants from moving. This immobility requires different developmental processes and mechanisms to cope with numerous environmental factors, such as light, gravity, water, nutrients, and temperature. The plant's ability to respond to its environment is affected by signaling messengers, called plant hormones. In animals, hormones orchestrate body functions by being produced in one place and acting in another. Plants have not a transport system as efficient as the blood circulation and no equivalent to the central nervous system for the coordination of all physiological activities. Nevertheless, plants show regulated growth, differentiation, metabolism, cell-to-cell communication, and other physiological activities, which are governed specifically by the abovementioned phytohormones.

Plant hormones are small molecules effective at very low concentrations, are usually synthesized in defined organs, and can be transported to other sites of the plant body, where they trigger specific responses. However, plant hormones may also act in the tissues in which they were produced[2]. As hormones can stimulate as well as inhibit plant growth, they are generally referred as plant growth regulators. The first discovered hormones were classified into five major groups: auxins, gibberellins, ethylene, cytokinins, and abscisic acid. More recently, regulation of plant growth, cell-to-cell communication, and plant immunity have been found to rely also on other molecules, including brassinosteroids, jasmonic acid, salicylic acid, strigolactones, nitric oxide, polyamides, and signaling peptides[3,4].

Plant hormones are distributed within the tissues, either from cell to cell (auxin), or via vascular bundles (cytokinin), or via the intercellular space (ethylene). Phytohormones interact with specific target tissues to trigger physiological responses, although each response is often the result of the combined action of two or more hormones. This phenomenon of hormonal crosstalk has been extensively studied in the recent years and the elucidation of molecular

mechanisms by which hormones interact to control growth and development contributes to a better understanding of basic plant physiology.

1 Auxin

Among all the plant hormones, auxin is the most important, because it controls many aspects of plant growth and various developmental processes, from cell division, cell elongation, cell differentiation to root initiation, apical dominance, tropism, flowering, fruit ripening, seed germination, and senescence. These processes are regulated by auxin-induced changes in gene expression[5,6] as well as by non-transcriptional auxin perception and response that mediate polarity establishment in multicellular plant organisms[7–11]. The balance between auxin metabolism, transport, and perception determines the auxin activity in a given cell at certain developmental context. Auxin has been suggested to act as an integrator of the multiple plant hormonal activities, indicating a broad and complex regulatory network of auxin interactions and feedback loops during plant development[12].

1.1 Auxin biosynthesis

The early discovery of auxin and its crucial role in plant development generated an extensive scientific interest. Although indole-3-acetic acid (IAA) had been recognized as the main and most potent native auxin already in 1935[13], the question how auxin is synthetized remained unanswered for more than 70 years. Previous biochemical and genetic studies suggested two separate major biosynthetic routes: one dependent and one independent of the precursor tryptophan (Trp) (reviewed in[14]).

The importance of Trp-dependent auxin biosynthesis has been proven as essential, not only for plant developmental processes, such as embryogenesis, flower development, vascular patterning, but also for hormonal crosstalk[15–18]. In contrast, the molecular components and the physiological functions of the proposed Trp-independent pathway, in which IAA could be produced from indole-3-glycerol phosphate or indole[19], are still unknown.

More recently, the discovery of the first complete auxin biosynthesis pathway in plants[20–22] revealed that in a simple two-step auxin biosynthesis, Trp, a precursor of auxin, is first converted into indole-3-pyruvic acid (IPA) by the TRYPTOPHAN AMINOTRANSFERASE

OF *ARABIDOPSIS*1 TRYPTOPHAN AMINOTRANSFERASE-RELATED (TAA1-TAR) enzyme family[18,23,24] (Fig. 1.A). In a second step, YUCCA (YUC) flavin monooxygenase catalyzes the direct conversion of IPA to IAA[20–22].

Primarily, auxin is synthesized in the young developing organs, such as leaves, shoot apical meristems, and developing fruits and seeds[25,26]. Subsequent findings have pointed toward local auxin production in very distinct cell types, such as the quiescent center (QC), root tissues, vasculature of hypocotyls and apical hooks as well as root and apical embryo meristems[23].

Local organ or cell-specific auxin biosynthesis could be achieved by selective expression of particular members of the *YUC* and *TAA* gene family. Indeed, different *YUC* genes have been shown to be responsible for auxin biosynthesis in shoots (*YUC1*, *YUC2*, *YUC4*, and *YUC6*) and in roots (*YUC3*, *YUC5*, *YUC7*, *YUC8*, and *YUC9*)[21]. Specific temporal and spatial patterns of *TAA* genes have been observed, although the same set of genes were found to regulate both root and shoot development[18,23].

Overexpression of each *YUC* gene leads to the characteristic auxin overproduction phenotypes, such as long hypocotyls[15,16]. Multiple loss-of-function *yuc* mutants display defects in embryogenesis, seedling growth, flower development, and vascular pattern formation[15,16] and have phenotypes similar to those of known auxin signaling and transport mutants[27–29]. In accordance with the auxin biosynthesis model, in which TAA and YUC proteins participate in the same auxin biosynthesis pathway[21], inactivation of *TAA* genes mimics the characteristic *yuc* mutant phenotypes[16,23]. Furthermore, knocking out of *TAA1* and two of its close homologs, *TAR1* and *TAR2*, phenocopies the developmental defects of the auxin mutants *monopteros* and *pinoid*[14].

Besides the above described auxin two-step biosynthesis, at least two other pathways have been proposed: the indole-3-acetamide (IAM) pathway and the indole-3-acetaldoxime (IAOx) pathway (also known as CYP79B pathway). The IAOx pathway is present only in a small group of plant species that possess the enzyme CYP79B to convert Trp to the IAOx intermediate[30,31]. IAOx is probably not a main auxin metabolic intermediate because only slight growth defects are occur by the complete inhibition of the IAOx production in *Arabidopsis*[31]. By contrast, the IAM pathway has been suggested to exist widely in plants, but IAM production and the physiological function of the pathway remain unclear[32], although the conversion of IAM to IAA by the *Arabidopsis* AMIDASE 1 (AMI1) has been demonstrated [33].

In summary, the two-step auxin biosynthesis pathway opens a new opportunity to manipulate auxin levels in plants, an important tool in basic research for crop improvement[34], which, in turn, might shed light to better understand the molecular mechanisms by which auxin controls plant growth and development.

1.2 Auxin transport

In plants, auxin is transported by two major distinct pathways: a non-polar, passive distribution through phloem and an active cell-to-cell polar auxin transport (PAT). Most of the auxin is transported rapidly via an unregulated flow in mature phloem over long distances[35]. The second, short-distance way, is slower and mediated by specific influx and efflux carriers that transport auxin in a cell-to-cell manner from the shoot towards the root. Based on the cellular localization of the auxin influx carrier AUX1, which allows phloem loading of auxin from the source (young leaves or flowers) and unloading in the sink (root), both of the routes appeared to be connected[35,36].

The cell-to-cell auxin transport model has been postulated as the chemiosmotic theory[37,38]. Depending on the different pH in the apoplast and symplast, two forms of auxin, which is weakly acid (*pK* is 4.7) can exist. In the acidic extracellular space (pH 5.5), part of the auxin becomes protonated and can, thus, as an electrically neutral lyophilic form (IAAH) freely enter the cells. In the cytoplasm (pH 7), auxin molecules dissociate in an hydrophilic anion form (IAA^-) and can no longer pass easily through the cell membrane. The exit of the auxin anions is mediated by active anion efflux carriers (reviewed in[39]). In addition, passive diffusion can be further facilitated by the uptake activity of H^+/IAA^- symporters of the AUX1/AUX1-Like (LAX) family[40]. Besides the above mentioned AUX1, three others auxin influx carriers, LAX1, LAX2, and LAX3 have been characterized. AUX1/LAX transporters belong to the amino acid permease family of plasma membrane proton symporters with specific developmental functions[40,41]. AUX1 is involved in the regulation of gravitropism, hypocotyl phototropism, root hair development, and phloem “loading” and, together with LAX3, regulates lateral root emergence, whereas the whole AUX1/LAX1-LAX3 family plays an important role in phyllotactic patterning[35,40–44] (reviewed in[45]).

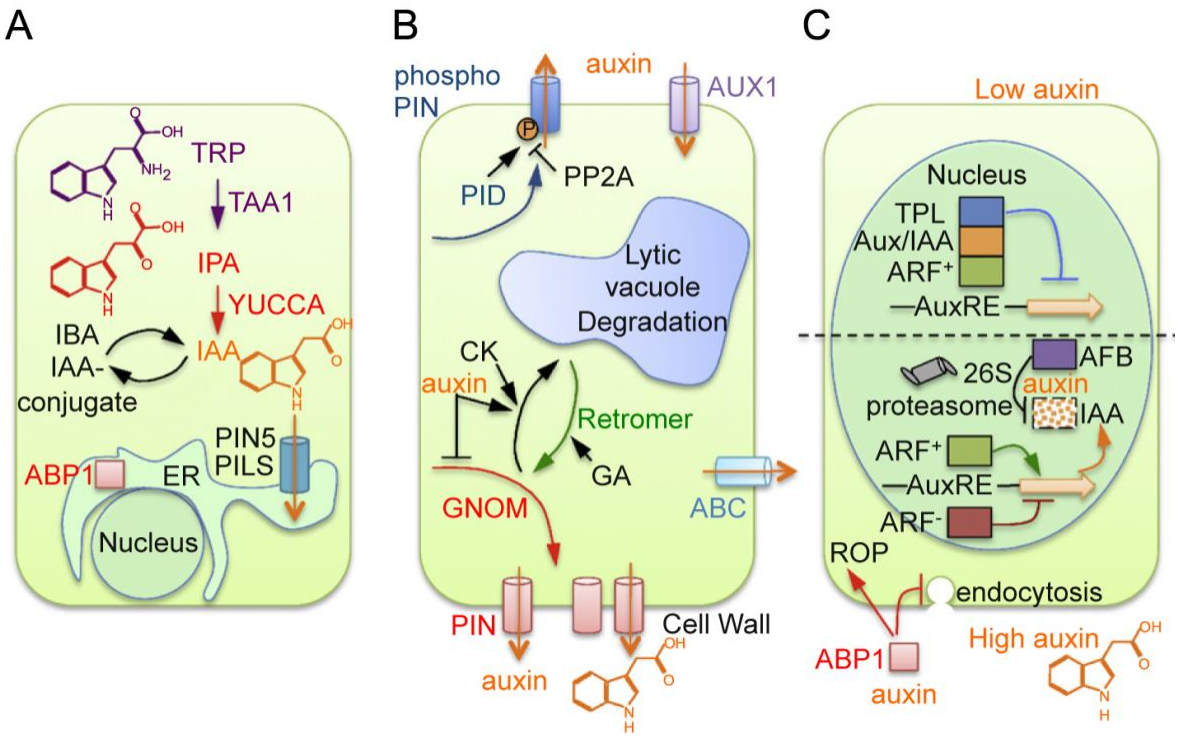

Figure 1. Schematic model of the auxin action in *Arabidopsis*. A. Auxin biosynthesis and homeostasis. Auxin is synthesized from the Trp precursor in a two-step pathway catalyzed by the enzymes TAA1 and YUCCA. PIN5 and PILS mediate the auxin flow from the cytosol into the lumen of the endoplasmic reticulum (ER) and thus regulate the intracellular auxin homeostasis. **B. Polar auxin transport and determinants of PIN polarity.** Polar auxin transport occurs in a cell-to-cell manner and depends on specific influx (AUX1) and efflux (PIN, ABC) carriers. PIN efflux carriers are constitutively recycled between the plasma membrane (PM) and endosomal compartments. PIN targeting to the basal PM requires GNOM activity, whereas apical targeting depends on the PIN phosphorylation status controlled by PINOID kinase (PID) and protein phosphatase 2A (PP2A). Auxin itself can inhibit clathrin-dependent endocytosis or promote PIN degradation in lytic vacuoles at certain concentrations. PIN vacuolar targeting is also induced by cytokinin (CK) signaling. The trafficking of PIN proteins to the lytic vacuoles occurs through prevacuolar compartments, from where they can be retrieved via the assistance of the retromer complex, possibly by promotion through gibberellins (GAs). **C. Auxin perception and signaling.** At low auxin concentrations, transcriptional repressors from the AUX/IAA family complex with the corepressor TOPLESS (TPL) dimerize with auxin response factors (ARFs) that remain transcriptionally inactive. At high auxin concentrations, auxin binds to its nuclear receptors from the TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX (TIR/AFB) family of the F-box proteins, which are subunits of SCF E3 ligase complex, leading to ubiquitination and proteasome 26S-mediated degradation of AUX/IAAs. Subsequently, de-repressed ARFs activate expression of auxin-regulated genes. Non-genomic rapid auxin responses are mediated via auxin perception through ABP1 receptor (adapted from[46]).

The contribution of other transporters in the auxin uptake has been demonstrated: the ABCB4 ATP-dependent auxin transporter acts in root epidermal cells when the auxin levels are low[47,48] and the NRT1.1 nitrate transporter facilitates uptake of auxin in roots under nitrogen deficiency[49]. Furthermore, ANT1 (aromatic and neutral amino acid transporter) is another possible candidate for auxin transport[50]. *ANT1* belongs to a small gene family of at least five members, exerts an auxin transport activity, and is produced everywhere with a

maximum abundance in flowers and cauline leaves. This expression pattern would be consistent with a role for ANT1 in auxin transport, probably through the vasculature. Taking into account the complexity of the auxin action in plant growth and development and the broad substrate specificity of ANT1, the physiological significance of ANT members need to be still characterized to prove their involvement in auxin transport activity. Auxin efflux is governed by four distinct classes of exporters: the PIN-FORMED (PIN) proteins[28], several phosphoglycoproteins (PGPs) from the ATP-binding cassette subfamily B (ABCB), members of the multidrug resistance (MDR) transporters[51–53], and, recently, the PIN-LIKES (PILS) proteins [54].

Among the auxin transporters, the PIN proteins have been proposed to be key rate-limiting components in the PAT[55,56]. The nomenclature of PIN proteins is derived from the pin-formed inflorescence phenotype caused by the loss of PIN1, the primary mediator of the directional auxin transport. In *Arabidopsis*, eight PIN proteins have been identified[28,57–60]. Based on the presence or absence of the central hydrophilic loop, they can be divided into two groups. The first group encodes full-length 'long' PIN proteins: PIN1, PIN2, PIN3, PIN4, and PIN7, which are localized at the PM and transport auxin out of the cell. Furthermore, PIN6, with only a partially reduced hydrophilic loop and high sequence similarity in the transmembrane regions, has been suggested to belong to the long PIN proteins[61], but, considering its physiological function and localization in the endoplasmic reticulum (ER) membrane, PIN6 is often classified into the group of short PIN proteins[45,62]. The short PIN proteins, comprising PIN5 and PIN8, lack the middle hydrophilic loop and are localized at the ER, where they mediate subcellular auxin homeostasis and compartmentalization[60]. The recently identified PILS proteins are also localized in the ER and are involved in the regulation of intracellular auxin homeostasis[54].

In addition to the PIN family of plant-specific auxin transporters, the auxin transport activity of ABCB1, ABCB4, and ABCB19 has been demonstrated both in plant and non-plant systems[52,55,63]. In contrast to the polar localization of PIN proteins that corresponds with the known auxin flow direction, the ABCB proteins act in a nondirectional, long-distance auxin transport that control the auxin amount in these streams[62].

All the members of the auxin transport machinery function coordinately to mediate auxin flows. In particular, the asymmetric distribution of PIN transporters at the corresponding cell sides determines the directionality of the auxin flow[64], which, in turn, establishes the local

auxin accumulation that is further supported by the local auxin biosynthesis[16,18,23] and plays a crucial role in auxin-mediated development[56].

1.3 Auxin transport regulation

Multiple aspects of auxin-dependent plant development are achieved by auxin transport and distribution within tissues. Auxin transport is governed by auxin influx and efflux carriers that can be controlled at many levels, including various endogenous and exogenous signals modulating, e.g. transcription, posttranslational modifications, protein degradation, subcellular trafficking, transport activity, PM composition, and interaction with other transporters (see below).

The transcription of carrier proteins, including PIN proteins, ABCB and AUX/LAX transporters, is regulated by auxin signaling components, such as the nuclear auxin receptor TRANSPORT INHIBITOR RESISTANT1 (TIR1)[47,63,65,66]. Auxin itself can induce transcription of PIN proteins through an AUX/IAA-dependent signaling cascade. This mechanism is tissue-specific and plays an important role in compensating loss of specific PIN proteins by up-regulation of other PIN family members[66].

Expression of PIN and AUX1 carriers is also modulated by other plant hormones, such as ethylene and cytokinin (CKs)[67–70]. Furthermore, both hormones regulate the expression of auxin-biosynthesis genes[69,71] and thus contribute to the local accumulation of newly synthetized auxin and its redistribution via polar transport.

Besides the transcriptional modulation of the auxin transport, CK-mediated regulation of PIN transporters occurs at the posttranscriptional level as well[72,73]. Endocytic trafficking of *PIN1* is enhanced from the PM to the lytic vacuoles after treatment with cytokinins[72].

The subcellular trafficking and targeting to specific positions on the PM represent the effective way to modulate PIN polarity. The current model proposes a two-step mechanism for the generation of the PIN polarity. In a first step, *de novo* synthesized PIN proteins are secreted from the Golgi apparatus to the PM in a non-polar manner. In the subsequent step, the polar localization of PIN proteins is established by their endocytosis and recycling[74].

Endocytosis depends on clathrin[75] and the PM sterol composition[76], whereas exocytosis is mediated by the endosomal protein GNOM, an ADP ribosylation factor-GTPase guanine nucleotide exchange factor (ARF-GEF)[77]. Additionally, the PIN recruitment into the apical or basal side of the cell is determined by its phosphorylation at defined residues[78–80]. Phosphorylation of PIN proteins by several kinases, including PID, targets these auxin

carriers to a GNOM-independent recycling pathway toward the apical pole of the cell, whereas dephosphorylation by PP2A leads to a GNOM-dependent basal targeting (Fig. 1B)[79,81]. Endocytosis and recycling define the PIN protein quantity at the PM by regulating the balance between recycling of the PIN proteins and their degradation in the lytic vacuoles (Fig. 1B)[82–84]. This balance is controlled by the retromer complex that consists of the SORTIN NEXIN 1 (SNX1) and VACUOLAR PROTEIN SORTING 26 (VSP26), VSP29, and VSP35 proteins[85] and could retain PIN proteins in prevacuolar compartments (PVCs), redirecting them back to the trans-Golgi network (TGN) and finally to the PM (Fig. 1B)[84].

Auxin itself seems to have a dual function in the regulation (of its own distribution) because it can inhibit clathrin-dependent endocytosis at certain concentrations or promote PIN degradation at others[10,82,86] (reviewed in[87]).

Besides the above-mentioned processes, the mechanisms underlying the proper establishment and maintenance of the PIN polarity are highly complex and are governed also by connections with the cell wall, the actin cytoskeleton, the phosphoinositide and calcium signaling, and the slow diffusion in the PM[88–91].

1.4 Auxin perception and signaling

The establishment of local auxin gradients, created by differential auxin distribution and local auxin biosynthesis leads to specific spatiotemporal patterns of auxin-mediated developmental responses[16,18,23,39,64].

The final developmental output of these patterns depends on the interpretation of the auxin signal via the auxin signaling pathway that, to a large extent, is mediated by the TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX (TIR/AFB) family of auxin receptors[29,92], the AUXIN/INDOLE ACETIC ACID (Aux/IAA) family of auxin repressors, the AUXIN RESPONSE FACTOR (ARF) family of transcription factors, and the transcriptional corepressor TOPLESS (TPL) (Fig. 1C)[93]. At low auxin concentrations, the Aux/IAA family forms a trimeric complex with the ARF transcription factors and the transcriptional corepressor TPL, thus preventing the ARFs from regulating the target genes (Fig. 1C, top)[93]. At higher auxin concentrations, auxin as a molecular glue between the TIR1/AFB and Auxin/Indole-3-Acetic Acid (Aux/IAA) transcription factors and stimulates their ubiquitination by $SCF^{TIR1/AFBs}$ E3-ubiquitin ligases and their subsequent degradation by

the proteasome, leading to derepression of ARFs and transcriptional regulation of the downstream response genes[29,92]. Recent findings showed that different combinations of TIR1/AFB and Aux/IAA proteins form coreceptor complexes with a wide range of auxin-binding affinities that might be determined mainly by Aux/IAAs because there are 29 Aux/IAA proteins and only six TIR1/AFB proteins in *Arabidopsis thaliana*[6]. The ARF family comprises 23 members that bind to an auxin response element (AuxRE) with the sequence TGTCTC in promoters of many auxin-responsive genes[94]. Sequence analysis and transient assays suggest that most of the 23 ARFs act as transcriptional repressors (ARF-) and only five (ARF5, ARF6, ARF7, ARF8, and ARF19) as activators (ARF+)[95]. Furthermore, most of ARF activators interact with the majority of the Aux/IAA proteins, whereas most ARF repressors do not or in a limited manner[96]. In addition, the limited interaction of ARF repressors imply that these transcription repressors might act independently of auxin and that they probably compete with the ARF activators for binding to the promoters of auxin-inducible genes (Fig. 1C). Therefore, the ratio between ARF+ and ARF- might influence the threshold of the auxin sensitivity in a given cell[46]. In addition to the extensively studied TIR1/AFB family, two other auxin receptors have been described: the AUXIN BINDING PROTEIN1 (ABP1)[97] and, more recently, the S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A)[98,99]. SKP2A is an F-box protein that forms a Skp, Cullin containing (SCF) complex in vivo which has E3 ubiquitin ligase activity[100]. SKP2A displays an auxin binding capacity, positively regulates degradation of the cell cycle transcription factors E2FC and DPB, and thus encompasses the direct auxin effect on cell proliferation and cell cycle control[100,101]. In contrast to SKP2A and TIR1, which are localized predominantly in the nucleus, most of the ABP1 is secreted in the lumen of the ER (99%) and small portion to the PM and extracellular spaces[102,103]. ABP1 signaling is involved in the regulation of rapid, non-genomic auxin effects at the PM[10,104]. As auxin binding to ABP1 inhibits clathrin-dependent PIN endocytosis (Fig. 1C), it promotes its own efflux[10,86]. This inhibition is mediated through the small GTPase of the RHO-OF-PLANT (ROP) family that acts downstream of ABP1 and is implicated in the formation and rearrangement of the actin and microtubule cytoskeleton. Moreover, existence of auxin-ROP2-PIN1-auxin positive feedback loop during lobe development[104], strengthening the role of auxin itself in PIN re-polarization that is essential for patterning and morphogenesis[9]. ROP proteins are also involved in other auxin-regulated processes, such as gravitropic responses and development of root hairs and lateral roots[105,106].

In conclusion, although the $SCF^{TIR1/AFB}$ signaling pathway regulates the expression of genes involved in many aspects of the auxin-mediated plant growth and development, a

number of fast cellular auxin responses occur within minutes independently of transcription. These rapid responses, such as auxin-induced cell expansion and proton secretion at the PM (reviewed in[99]), cytoskeleton rearrangements[104] (Fig. 3), and inhibition clathrin-mediated endocytosis[10], happen within the individual cell and affect the spatial and temporary auxin distribution. Together with the transcriptionally regulated expression of auxin-inducible genes, a wide range of developmental processes are governed in response to auxin signals.

1.5 Auxin as key player in plant development

Local auxin biosynthesis together with PAT generates local auxin gradients that are further controlled by genomic and non-genomic auxin mediated responses. The establishment of these gradients plays important roles in various aspects of plant growth and development, including responses to environmental stimuli such as light (phototropism) and gravity (gravitropism)[59,107], and multiple processes necessary for the regulation of *de novo* organogenesis[108], root meristem activity[109], vascular tissue differentiation[110], and female gametophyte[111]. Hence, an auxin gradient is created in cells in which auxin accumulates, triggering a change in the developmental program. However, auxin does not necessarily have to form an auxin gradient to induce morphogenetic events, but can act more like a threshold-specific trigger above or under a certain limit concentration[112], thus, creating an auxin maximum[113] or minimum[114].

Whereas the formation of a local auxin minimum has been demonstrated to date only in one particular event, namely the specification of the valve margin separation layer during the fruit opening[114], a wider spectrum of developmental processes is coordinated by the creation of auxin maxima. In early embryogenesis, auxin maxima regulate the establishment of the apical-basal axis of root pole and cotyledons[115], as reflected in the postembryonic development, in which the auxin maxima mark the organ initiation positions[116] and, subsequently, the tips of developing organ primordia[108]. After primary root and lateral roots (LRs) become functional, the auxin maxima are grounded into the quiescent center (QC) and columella cells and play a crucial role in the maintenance of the normal meristem organization and function[58,109].

Establishment of auxin maxima and minima is mediated by the coordination of auxin fluxes and distribution at the tissue level. To understand the connection between cellular and

tissue polarity, a canalization model has been proposed. In that model, auxin acts as a polarizing impulse and the auxin flow through the tissues will amplify existing auxin fluxes and polarize neighboring cells. Auxin exerts a feedback effect on its own transport[117]. The molecular mechanism underlying the proposed model has been observed in *de novo* formation and regeneration of vascular tissues and venation patterns[118,119]. Taking into account the auxin feedback on PIN endocytosis[86] and on *PIN* transcription[120], a novel mechanistic framework for canalization has been proposed, predicting the existence of extracellular receptors[121]. Although this extracellular receptor-based polarization model proposes a mechanistically feasible explanation for the PIN polarization and canalization of auxin fluxes, these predictions have to be validated experimentally.

1.6 Auxin as a trigger of embryogenesis

During early embryogenesis, the apical-basal and radial body axes are formed, followed by the establishment of bilateral symmetry through the auxin maxima that serve as reference for postembryonic development. The apical–basal axis specification depends on dynamic changes in auxin accumulation and fluxes (reviewed in[122]). After the first anticlinal zygotic division, an asymmetric distribution of the PIN7 efflux carrier directs the auxin flow into the apical cell, resulting in increased auxin accumulation[115]. The auxin transporters PIN1, ABCB1, and ABCB19 contribute to the auxin transport during this initial stage[115,123]. Subsequently, at the globular stage, PIN1 relocalizes basally in the pro-embryonic cells and PIN7 shifts to the basal PM of suspensor cells. PIN4 is also involved in this polarity relocalization that, in turn, results in an auxin flow directed toward the embryo top through the protodermal layer and then toward the root pole (Fig. 2)[108,115], leading to auxin accumulation in the hypophysis, a root meristem precursor. At the heart stage of the embryo development, the PIN1 activity defines three zones: auxin gradients in the epidermis mark the position of cotyledon primordia, auxin flow downward through the center determines the future vasculature, and the zone depleted in auxin represents the future shoot apical meristem (SAM) (Fig. 2)[108]. Besides the auxin flow, the importance of the local auxin synthesis to generate auxin maxima can be deduced from the observation of severely defective embryos and cotyledons in multiple *yuc* mutants[16,22,23].

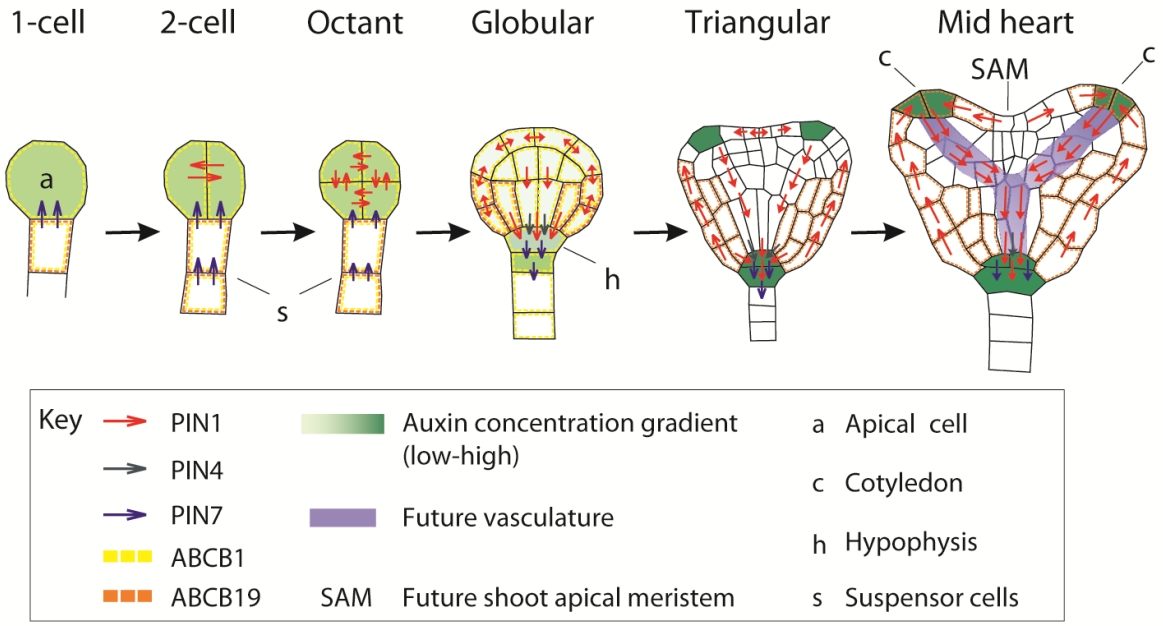

Figure 2. Differential auxin distribution during embryogenesis. After division of zygote, PIN7 localizes to the apical side of the basal cell directing auxin transport to the apical cell. PIN1 is localized apolarly from the one-cell to globular stage and together with ABCB1 and ABCB19 distribute auxin homogenously. At the globular stage, PIN7 polarity reverses to the basal membrane of suspensor cell, PIN1 becomes localized basally in the pro-embryo cells and together with activity of PIN4 transport auxin to the hypophysis. During the triangular and heard stage polar localization of PIN1 in the epidermis generate auxin maxima at the sites of cotyledon initiation and the bilateral symmetry is established (adapted from[45]).

Local auxin gradients activate the transcription factors MONOPTEROS (MP)/ARF5 and NONPHOTOTROPIC HYPOCOTYL4 (NPH4)/ARF7 by degrading the transcriptional inhibitor IAA12/BODENLOS (BDL)[124]. The MP activity is essential for hypophysis specification that initiates the primary root meristem formation[125].Downstream targets of MP are the PLETHORA (PLT) transcription factors that are essential for QC specification and positioning of the stem cell niche to the basal side of the embryo[126]. *PLT* genes can, in turn, induce the expression of *PIN* genes that establish the auxin maxima in the root meristems[127].

MP binds also to the promoter of the transcription factor DORNRÖSCHEN (DRN). Hence, auxin (through MP) regulates directly and positively the *DRN* expression in the cotyledon tips of torpedo embryos[128]. Loss-of-function *drn* mutants display a disrupted and diffuse DR5 activity and an altered polar localization of the *PIN1* gene in embryos[129], indicating that DRN acts upstream of the auxin transport.

Local auxin concentrations play also a role in establishing the primary shoot meristem by influencing the expression patterning of key transcription factors in the apical region of the embryo. The expression patterns of class I *KNOTTED-LIKE HOMEODOMAIN* (*KNOX*)

genes, such as *SHOOT MERISTEMLESS* (*STM*) and *CUP-SHAPE COTYLEDON* (*CUC*), overlap with low auxin levels[130,131].

Taken together, the spatiotemporal auxin maxima and specific expression patterns of auxin-responsive genes and other patterning genes determine the apical-basal and radial polarities and developmental potential.

1.7 Auxin and postembryonic development of lateral organs

Auxin is not distributed homogeneously at the emerging positions of the primary root, shoots, and cotyledons during embryogenesis. In postembryonic development, auxin maxima indicate the organ initiation positions and later the tips of the developing organ primordia[108]. In this context, auxin maxima and directional auxin flow are crucial regulators in postembryonic development of lateral shoot and root organs and are described by the fountain and reverse-fountain model[108] (Fig. 3A).

Directionality of auxin flow is determined by the coordinated action of auxin transport systems. The simplified scheme of their actions include the rootward auxin flow in the stele primarily mediated by PIN1 and ABCB19[63,132], the shootward auxin transport from the root apex mediated by AUX1, PIN2, and ABCB4[35,47,133,134], whereas the auxin motion from the shoot meristem is regulated by PIN1, PIN7, ABCB1, ABCB19, and AUX1 and from the root meristem by PIN3, PIN4, AUX1, ABCB1, and, to a lesser extent, ABCB19[27,52,135,136]. PIN7 significantly contributes to auxin maintenance in meristems[27].

LRs branch from the primary root and their initiation begins when the pericycle founder cells (FCs) undergo several rounds of anticlinal divisions that form the LR primordium from which the LR will emerge[116,137]. Potentially, every pericycle cell has the ability to divide in response to increased auxin levels[138], but only a few act as FCs. Indeed, auxin regulates the spacing of the pericycle FCs by creating local auxin accumulations in the protoxylem that prime the adjacent pericycle cells to become FCs[139].

Each step in the process of developing LR primordia (LRP) is in accordance to a highly regular pattern controlled by auxin via a number of ARFs and their AUX/IAA inhibitors[140,141]. Local auxin accumulation promotes the degradation of the SOLITARY ROOT (SLR)/IAA14, causing derepression of ARF7 and ARF19 that, in turn, directly activate the expression of the cell cycle transcription factors ASYMMETRIC LEAVES2-LIKE/LATERAL ORGAN BOUNDARIES-DOMAIN16 (LBD/ASL16) and LBD/ASL18 and control the initial

asymmetric pericycle cell divisions[65,141,142]. Auxin responses mediated by the MP/ARF5-BDL, which acts downstream of SLR/IAA14, control the organized LR patterning in asymmetrically dividing pericycle cells[143]. During LR emergence, increased auxin accumulation in dividing pericycle cells induces degradation of the SHY2/IAA3 repressor, leading to the expression of cell-wall-remodeling genes. Subsequently, cells overlaying the LRP undergo cell separation that allows emergence of primordia[40].

Besides auxin signaling, auxin transport is also crucial for LR initiation and development (Fig. 3B). Upon the establishment of FCs, the auxin-induced expression of *PIN3* in the lateral inner endodermis membrane mediates the auxin reflux and accumulation in the FCs, which is important for the transition from FCs to LR initiation[144]. Along with the first anticlinal division of pericycle cells, followed by periclinal divisions, PIN1 is relocalized from the anticlinal membranes of the pericycle cells to the outer lateral PMs of inner cells[108]. The molecular mechanisms underlying the switch in PIN1 polar localization involve GNOM-dependent transcytosis[145], leading the auxin flow toward the primordium tip, where an auxin maximum is formed[108,146]. In addition, auxin transport during LR formation is facilitated by ABCB1,19 transport activity, auxin availability in AUX1-governed LR initiation regions, epidermal auxin uptake by LAX3 during emergence, and PIN2-mediated auxin flux away from the new formed tip at the later stages of LR development[35,40,52,55,123] (reviewed in[45]) (Fig. 3B).

In conclusion, auxin is essential, not only for LR patterning[140,147], but also for induction of reprogramming cells overlaying the LRP to facilitate organ emergence[40] and possibly for the establishment of new meristems of emerging primordia and their elongation[140].

Shoot lateral organs are formed from the organ FCs, of which the initiation results from two antagonistic processes mediated by local transcription networks. First, the transcription factors STM and CUC3 determine the meristematic identity[148]. Second, once a new organ is initiated, the transcription factor ASYMMETRIC LEAVES1 (AS1) represses the meristem identity factor STM in the developing primordia[149]. The meristem generates either leaves and lateral meristems or flowers. The identity of the lateral organs produced depends on the activity of LEAFY (LFY)[150,151]. Another early marker of organ initiation is the transcription factor AINTEGUMENTA (ANT), which is highly expressed in the initiated organs and acts via regulating genes involved in cell proliferation, such as the D-type CYCLIN[152]. This complex transcriptional network has been proposed to be coordinated by auxin, including its transport, biosynthesis, and signaling (Fig. 3C) (reviewed in[153]). Application of auxin

promotes the formation of leaves and flowers[154] and that auxin is distributed asymmetrically at the SAM with auxin maxima at the emerging primordia positions[108].

Formation of local auxin maxima is mainly maintained by the PIN1 action at the meristem surface that transports auxin through the epidermal layer toward the primordium tip[108]. Additionally, AUX1, LAX1, LAX2, and LAX3 transporters regulate organ positioning by ensuring auxin delivery to the epidermal layers[42].

PIN1 localization and polarity at the PM depends on PIN trafficking to the PM modulated by the PID kinase activity[79,81]. *PID* is strongly expressed at the boundaries that separate organs from the meristem, where *PIN1* undergoes complex polarity rearrangements indicating that PID may play an important role in organ separation[130,155].

Although not much is known about the regulation of auxin signal transduction in the shoot meristem, expression of *MP*/*ARF5* at the meristem margins[156], suggests that lateral organ initiation depends also on a spatial modulation of auxin signaling.

The importance of local auxin biosynthesis in organ initiation has been revealed by the analysis of multiple loss-of-function *yuc* mutants that exhibit defects in inflorescence, floral, and leaf vasculature development[15]. Taken together, these results imply that local auxin concentrations in the shoot meristem are controlled by PAT and auxin biosynthesis.

As in other tissues, SAM is regulated by the coordinated action of several hormones, including CKs and gibberelins[157–159]. Callus regeneration assay have revealed that a high cytokinin to auxin ratio induce the initiation of shoot meristems from undifferentiated callus[160]. To date, extensive crosstalk between these two hormones has been reported in many aspect of plant development on multiple levels including metabolism, signaling, transport, transcription and post-transcriptional control. Therefore, to unravel this complex network between auxin and CK, first CK metabolism, transport, and signaling will be discussed in the following sections.

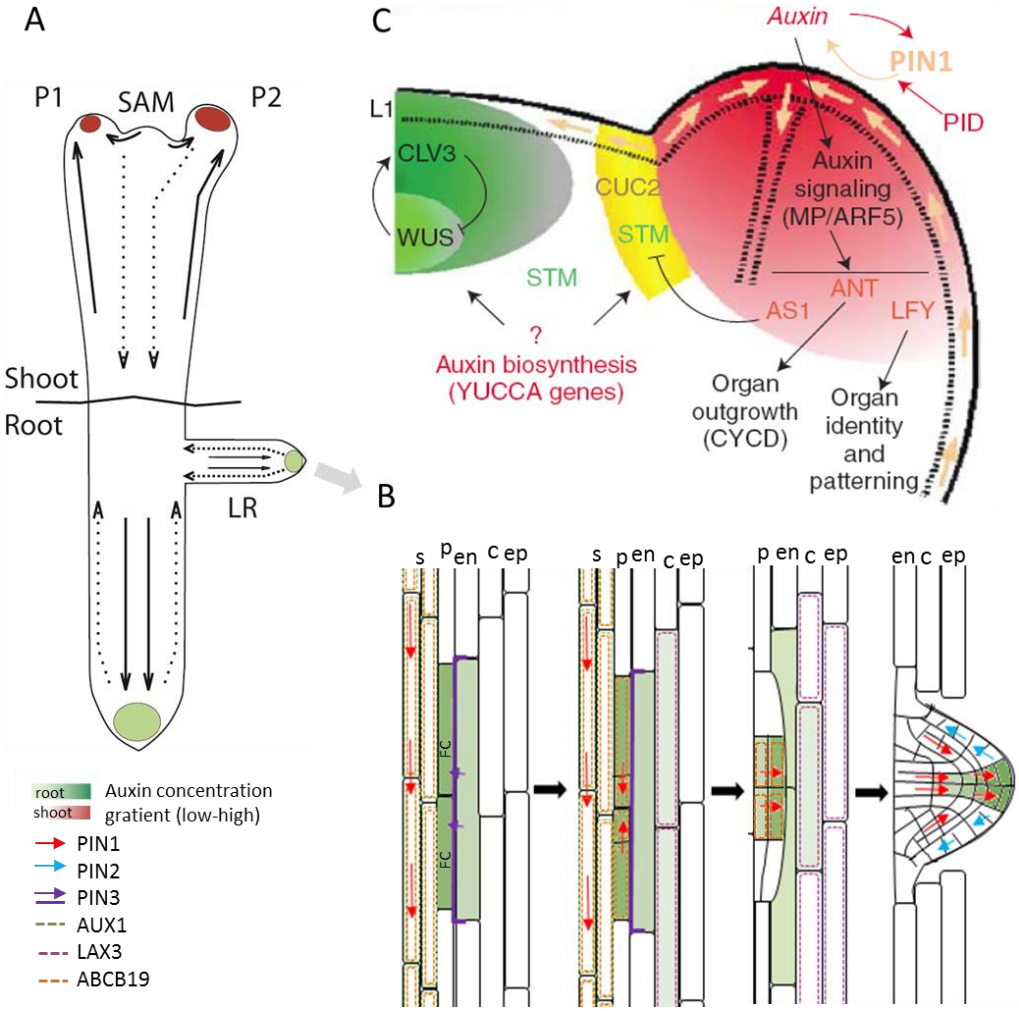

Figure 3. Role of auxin during organogenesis. A. Fountain and reverse-fountain model. Acropetal auxin transport to the tip of particular organs (root, shoot, and lateral organs) (solid arrows) together with reverse fluxes (dashed arrows) from the tip toward the basis of shoots and roots regulate the establishment and maintenance of the auxin maxima in lateral organs. Acropetal transport of auxin in the shoot and leaf primordia (P1 and P2, red) is accomplished through epidermal layers and refluxed back through the future vasculature. In the root and LRs (green), auxin is transported toward the tip through the internal part of the primordium and refluxed back through the epidermal layer **B. Auxin transporters during LR development.** Auxin response activity in a restricted number of pericycle cells indicates funder cell (FC) establishment. PIN3, localized laterally to the inner membrane of the endodermis cell, facilitates auxin transport towards the founder cells. This provides a local auxin reflux circuit which contributes to auxin accumulation in FCs important for further progress to LR initiation (LRI). During LRI, PIN1 is localized at the anticlinal membranes. In concert with the division plane switch, PIN1 is lateralized. Later, in dividing primordia, auxin is transported from the tip through the outer epidermal layers via PIN2. The transporters AUX1, ABCB19 control the auxin supply in the root tip. LAX3 is involved in auxin-mediated induction of cell-wall-remodeling genes, which are essential for organ separation and LR emergence. C, cortex; en, endodermis; ep, epidermis; p, pericycle, s, stele. **C. Role of auxin during shoot-derived lateral organs.** SAM patterning and maintenance is controlled by the WUSCHEL (WUS)-CLAVATA3 (CLV3) negative feedback loop. Lateral organ initiation is mediated by the action of the transcription factors STM, CUC2, ASYMMETRIC LEAVES1 (AS1), and ANT, whereas LEAFY (LFY) is involved in lateral organ specification. Auxin maxima are maintained by local auxin biosynthesis and polar transport. Transport through the outer epidermal layer (L1) is mediated by PIN1, of which the polarity is controlled by PID. Expression of MP in the lateral organ boundary hints at a role of auxin signaling at initial stages of the lateral organ development (adapted and modified from[45,96,144]).

2 Cytokinins

Cytokinins have been identified in almost all higher plants as well as in mosses, fungi, bacteria and in tRNA of many prokaryotes and eukaryotes[161]. Like auxin, CKs also play a major role in various aspects of plant growth and development including cell division, regulation of root and shoot growth and branching, leaf senescence, chloroplast development, seed germination, stress tolerance, and pathogen resistance[162]. These processes are governed by downstream components of the CK signaling pathway after its perception and signal transduction. In contrast to auxin, the CK signaling pathway in plants represents a two-component signaling system, a canonical mechanism that mediates diverse biological responses in many *taxa*[163,164]. The limiting step of the whole machinery is the availability of the active CK at certain concentrations on the right place at the right time to interact with specific receptors. The levels of active CKs are spatially and temporally regulated by a fine balance between biosynthesis, transport, and degradation[163]. Changes in CK levels have been shown to affect various aspects of plant development[165–168]. Interplay of CK metabolism, transport and signaling, as well as interactions of CKs with other plant hormones confer these hormones the capacity to coordinate their multiple functions throughout the plant life.

2.1 Cytokinin metabolism

The naturally occurring plant CKs are adenine derivatives with an isoprenoid side chain. Isopentyladenine (iP) and *trans*-zeatin (tZ) are the most common active CKs in *Arabidopsis*[169], whereas *cis*-zeatin (cZ) predominates, for instance, in rice[170] (*Oryza sativa*) and maize (*Zea mays*)[171] (reviewed in[161]). iP and tZ have been demonstrated to bind as ligands to the three CK receptors *ARABIDOPSIS* HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4/CYTOKININ RESPONSE1 (CRE1)[172–174].

CKs are abundant in root tips, shoot apices, and immature seeds, where their synthesis is also assumed to take place[166,175]. CK biosynthesis is achieved in a multistep process (Fig. 4). In the initial rate-limiting step of iP and tZ biosynthesis, the isoprenoid group is transferred by isopentyltransferases (IPTs) to the N6 atom of AMP, ADP, or ATP to generate iP-ribotides[176,177]. Isopentenylated precursors of CK are subsequently hydroxylated by cytochrome P450 monooxygenase A (CYP735A) to tZ-ribotides[178]. Biosynthesis of cZ is initiated by tRNA-IPTs that catalyze the isopentenylation of adenine in tRNA[177]. However,

the enzyme for *cis*-hydroxylation has yet to be identified in plants. After dephosphorylation of iP-, tZ-, and cZ-ribotides, conversion of iP-, tZ-, and cZ-riboside 5-monophosphate to their active forms is catalyzed by the CK-specific phosphoribohydrolase LONELY GUY (LOG)[179,180].

The irreversible degradation of CK is mediated by the CK oxidases (CKXs) that are responsible for most metabolic CK inactivation in many plant species[162]. CKXs selectively cleave unsaturated *N6* side chains from tz and iP and their corresponding ribosides, resulting in inactive CK products[181].

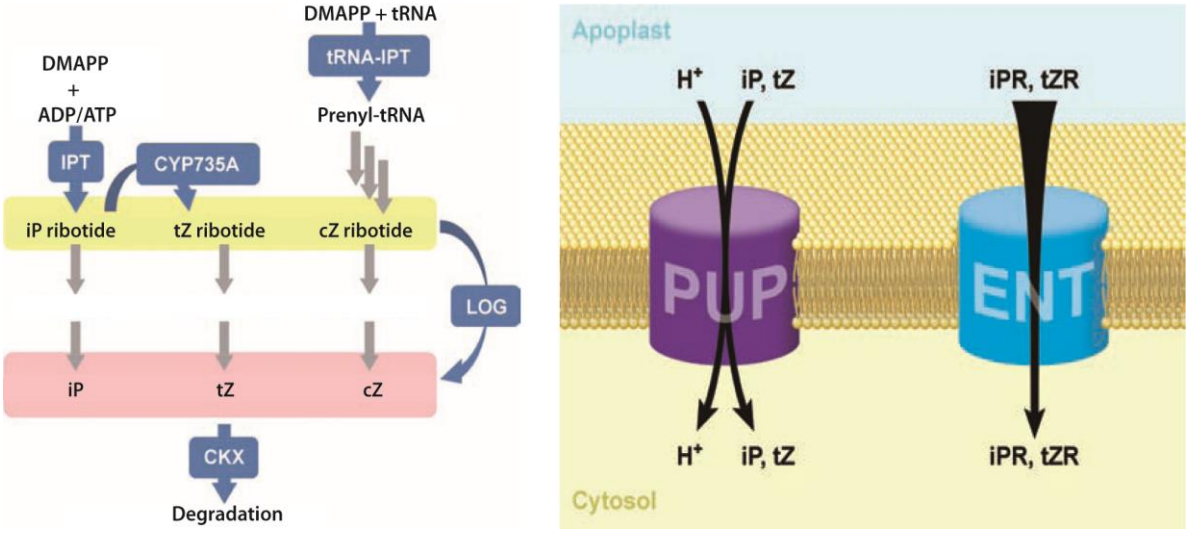

Figure 4. **Core steps of CK metabolism** (left) **and schematic model of CK transport across the PM** (right). Biosynthesis of iP-CKs and tZ-CKs is initiated by adenosine phosphate-isopentenyltransferases (IPTs) to form iP-nucleotides that can be converted to the corresponding tZ-nucleotides by cytochrome P450 monooxygenases (CYP735As). *cis*-Zeatin (cZ) CKs, which are the major CK metabolites in some plant species, are synthesized in *Arabidopsis* exclusively by tRNA-IPTs that utilize tRNAs as prenyl acceptors. iP-, tZ-and cZ-ribotides can be dephosphorylated to the corresponding iP-, tZ-and cZ-ribosides (iPR, tZR, cZR), or directly converted to active free bases by CK nucleoside 5'-monophosphate phosphoribohydrolases (LOGs). *Arabidopsis* PURINE PERMEASE 1 (AtPUP1) and AtPUP2 can transport iP and tZ, in a proton-coupled manner, whereas diffusion of CKs may be additionally supported by uptake activities of the Equilibrative Nucleoside Transporter (ENT) family proteins that transport CK ribosides, such as iPR (adapted from[182]).

Genes encoding CK biosynthesis and degradation enzymes are, with the exception of *CYP735A*, widely expressed throughout the plant, although some of them exhibit spatial specificities (Fig. 5). In *Arabidopsis*, there are nine *IPT* genes[183], of which seven (*IPT1*, *IPT3*, *IPT4*, *IPT5*, *IPT6*, *IPT7*, and *IPT8*) from the ATP/ADP IPT class are predominantly expressed through the root[184]. However, some *IPT* genes have specific expression patterns; e.g., *IPT1* is expressed in the xylem, *IPT5* in the root primordia and columella root cap, and *IPT7* in the

endodermis at the elongation zone[184]. Two genes encoding tRNA-IPTs (*IPT2* and *IPT9*) are expressed ubiquitously with the highest expression in proliferating tissues[184].

Mutants lacking *IPT1*, *IPT3*, *IPT5*, and *IPT7* showed reduced levels of tZ and iP together with inhibited shoot growth, whereas the overall root growth was enhanced[177]. Genes encoding two CYP735A, identified in *Arabidopsis,* are predominantly expressed in roots and induced by CKs[178,185]. Hence, CK-dependent induction of *CYP735A* expression might lead to changes in the metabolic flow and storage of redundant CKs in an inactive form[178].

In *Arabidopsis*, nine *LOG* genes (*LOG1* to *LOG9*) had been predicted to be rice *LOG* homologs, of which seven (*LOG1, LOG2, LOG3, LOG4, LOG5*, *LOG7*, and *LOG8*) possess phosphoribohydrolase activity[186]. The expression patterns of *LOG* genes are spatially and quantitatively differentiated, but overlap in various tissues during plant development, hinting at redundant functions for the *LOG* gene family members and CK activation in nearly all parts of the plant[179,186]. Analyses of the loss-of-function mutant *log3log4log7* suggested that *LOG* genes control the CK activity during normal development in *Arabidopsis*[180,186].

CK degradation enzymes in *Arabidopsis* are encoded by a family of seven *CKX* genes[187]. Similarly to *IPT* and *LOG* genes, *CKX* genes are widely expressed, but some of them have unique spatial expression pattern (Fig. 5). *CKX1*, *CKX2*, and *CKX6* are expressed in the central cylinder, whereas *CKX1* is also expressed in the shoot apex and pericycle around the LR branching point. Expression of *CKX4* and *CKX5* occurs in the root cap and root apex, and *CKX5::GUS* activity is also detected in LRP[168]. Transgenic *CKX*-overexpressing plants exhibited decreased levels of CK, resulting in phenotypes indicative of CK deficiency, such as altered shoot formation and enhanced root growth[168] . The spatial distribution of CK metabolism enzymes together with the observation that various CK species accumulate in xylem and phloem[188,189] suggest that *de novo* CKs are produced in a tissue- and cell-specific manner and, thus, must be moved to target cells by diffusion and/or selective transport.

2.2 Transport of cytokinin

Like auxin, cytokinins are highly mobile in the plant system (reviewed in[190]). Compared with the well characterized transport machinery of auxin[35,38], the nature of the CK transport is less clear. An initial hypothesis regarding the movement of CKs from root to shoot had proposed a diffusion mechanism for CK uptake, based on the high permeability of the cell membranes to free CK bases and ribosides[191].

Study of radiolabeled tZ uptake in *Arabidopsis* cell cultures hinted at proton-coupled high-, medium-, and low-affinity CK transport systems[192]. So far, the *ENT* gene family and the purine permease (PUP) family have been proposed as candidates as CK transporters (Fig. 4)[193–196].

Among the *Arabidopsis* PUP protein family[197], the active uptake of tZ and iP by PUP1 and PUP2 had been demonstrated with a yeast system[193] and the expression of *PUP2* in the phloem of *Arabidopsis* leaves with promoter-GUS reporter fusions. This result, together with the expression of *IPT3* in phloem tissue[184] and the discovery of CKs in phloem sap[198], might point to a role for PUP2 in phloem loading and unloading for long-distance transport of adenine and, possibly cytokinins. Genetic studies on plant PUP proteins with loss-of-function or gain of-function mutants might help obtain further information regarding the CK transport and its function *in planta*. Competitive uptake studies in yeast cells showed that *Arabidopsis* ENT3, ENT6, ENT7 and rice ENT2 can uptake iPR and tZR (Fig. 4)[194,195]. Furthermore, mutants lacking either *ENT3* or *ENT8* exhibit a reduced CK uptake efficiency[196].

Distinctive expression patterns of *ENT3* and *ENT8*[194,196] suggest that they may act differently during plant growth and development, whereas *ENT6::GUS* is expressed in root, leaf, and flower vasculatures[198], indicating the involvement of *ENT6* in long-distance transport.

Long-distance transport of cytokinins is supported by the discovery of CKs in xylem and phloem sap and by grafting experiments between wild-type and CK biosynthesis mutants. In xylem sap, the major form of CK is tZR[198–200], whereas in phloem sap iP-type CKs, such as iPR and iP-ribotides, are the major forms[198,201]. Thus, it is conceivable that plants might use tZR as an acropetal and iP-type CKs as basipetal messengers[182]. Reciprocal grafting experiments between *ipt1, ipt3, ipt5,* and *ipt7* mutants and wild-type plants showed a preferential transport of different CK species: tZ-type CKs were transported from the root to the shoot, whereas iP-type CKs moved from the shoot to the root[202]. Acropetal transport of CKs (toward the shoot apex) has been implicated in the control of shoot branching as well[203]. Basipetal transport of CKs through the phloem has recently been demonstrated with the direct transport to the root tip of radiolabeled CK applied to hypocotyls. Moreover, lines with impaired phloem transport have revealed that a basipetal CK translocation occurs through symplastic connections in the phloem[204].

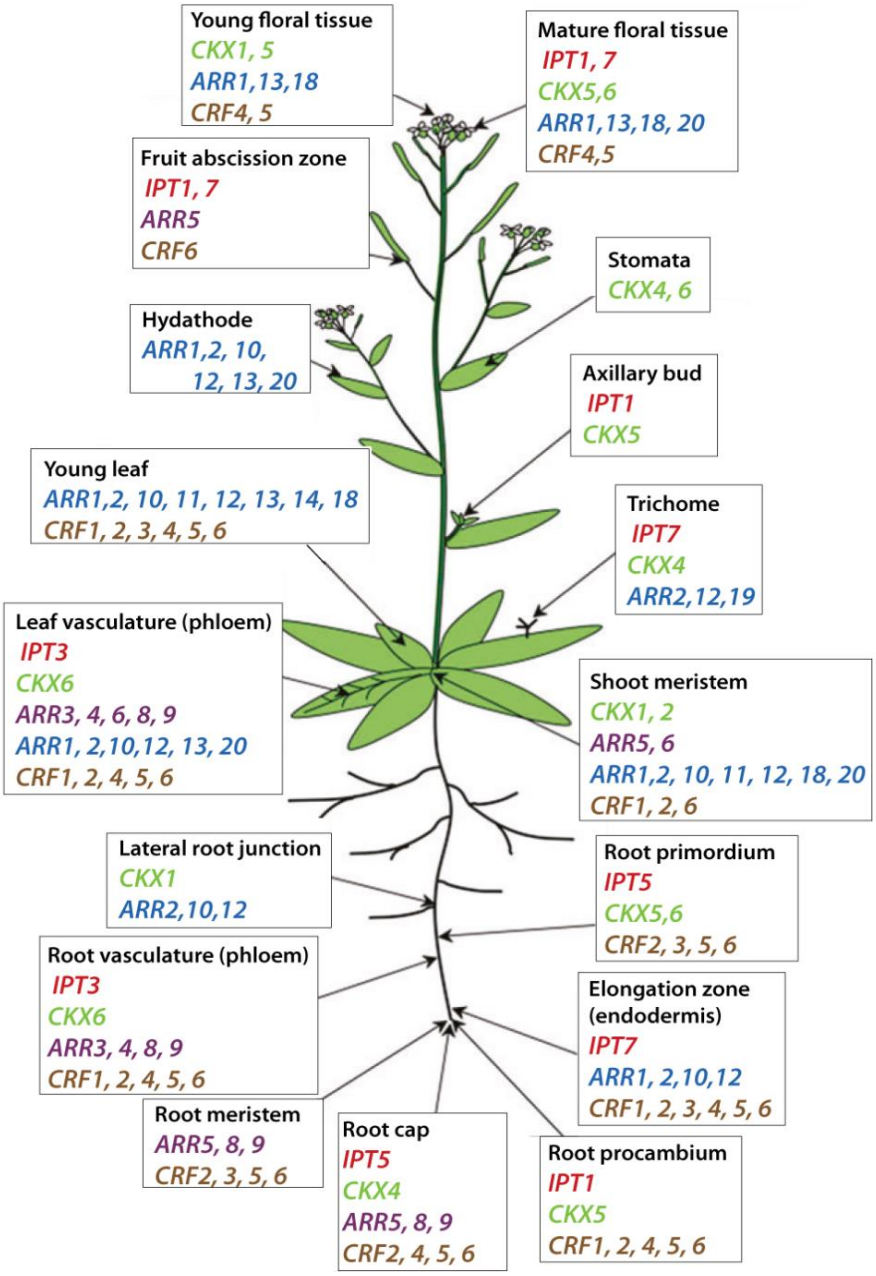

Figure 5. Spatial expression of genes involved in CK metabolism and signaling. Data are derived from studies of promoter-reporter gene (GUS and/or GFP) fusions in transgenic *Arabidopsis* lines harbouring cytokinin biosynthesis- *IPT* genes (red)[184,207], degradation-*CKX*s (green)[168,208] and signaling- type A ARRs (violet)[209–213], type B ARRs (blue)[214,215] and CRFs (braun) [216,] (Chapter 4 and unpublished data) (adapted and modified from[198]).

In summary, characterization of rate limiting enzymes of the CK biosynthesis and their spatial expression patterns (Fig. 5) suggests that local transport and biosynthesis play important roles in the regulation of developmental processes[177,187]. Recent reports provided evidence for the presence of CK receptors in the ER[174,205,206], emphasizing the importance to identify CK transporters and their subcellular localization, which, in turn, may lead to the

discovery of new regulatory mechanisms in this subcellular compartment. Furthermore, the discovery of symplastic connections for basipetal CK transport in the phloem[204] demonstrated that long-distance delivery of CKs through the phloem enables the targeting of specific developmental processes and crosstalk with other signaling pathways.

2.3 Cytokinin signaling and perception

Cytokinin activity and availability in specific organs or tissues is regulated by the orchestrated action of metabolism and translocation. The interaction of active CKs with specific receptors, the signal transduction to downstream components, and the effector proteins together with the spatial distribution of CK signaling components (Fig. 5) determine the output responses and the domains in which cytokinin response occurs.

Identification of major components of CK signaling has revealed that this mechanism is similar to two-component signaling (TCS) found in bacteria, consisting of the sensor histidine kinase (HK) proteins, the histidine phosphotransfer (HPT) proteins, and the effector response regulator (RR) proteins (Fig. 6A)[163].

Binding of active CKs to the Cyclases/Histidine kinases Associated Sensory Extracellular (CHASE) domain of the HK receptor induces its autophosphorylation at a conserved histidine (His) residue in the N-terminal sensor kinase domain, resulting in the transfer of a phosphoryl group to an aspartate (Asp) residue in the C-terminal receiver domain. The phosphoryl group is then carried over to a conserved His on a histidine phosphotransfer (HP) protein that ferries the phosphate to the nucleus on an Asp in the receiver domain of a response regulator (RR) (Fig. 6E)[217].

2.3.1 Signal sensing via histidine kinases

Three transmembrane sensor HKs have been identified as CK receptors in *Arabidopsis*: AHK2, AHK3, and AHK4/CRE1/WOODEN LEG (WOL)[218–220]. Two other *Arabidopsis* HKs, CYTOKININ INDEPENDENT 1 (CKI1) and CKI2 (also known as AHK5) that do not contain the CK-binding CHASE domain, can also activate CK signaling in the absence of exogenous CKs[221,222]. Constitutively active CKI1 regulates vascular tissue development in shoots[223] and is crucial in the process of female gametophyte development because loss-of-function mutants display female gametophytic lethal phenotypes[224,225]. AHK5 has been

suggested to act as a negative regulator of the signaling pathway in which ethylene and abscisic acid inhibit the root elongation through the ethylene receptor ETHYLENE RESPONSE 1 (ETR1)[221,226]. Yet, the molecular mechanism underlying the function of AHK5 in these processes remains unknown. Taken together, despite emerging evidence that *CKI* genes contribute to the CK signaling independently from AHKs, it remains to be determined whether the CKI activity directly affects the CK signaling or other TCS-mediated responses.

In contrast to CKIs, AHK2, AHK3, and AHK4 require CK for their activation[173,218,227], but they exhibit varying sensitivities to different CK species, possibly hinting at their differential involvement in specific signaling pathways[173,228]. The large overlap in expression patterns and high conservation in protein structures of the AHK2, AHK3, and AHK4 receptors have resulted in genetic and functional redundancy between their loci[175,229]. This is in accordance with the only subtle observed phenotypes of the CK receptor single mutants (*cre1*, *ahk2*, and *ahk3*) compared to the strong phenotype of a triple mutant lacking all three receptors. In various assays, triple CK receptor mutants showed no response to CK and produced small infertile plants with reduced meristem sizes[175,229]. Furthermore, a loss of the CK receptors resulted in female gametophytic lethality [230].

Recent studies have presented evidence that a large majority of the CK receptors are present on the ER membrane (Fig. 6E), implying that this compartment might play a key role in CK signaling[205,206,231]. The internal localization of the receptor, suggesting the need for CK perception within the lumen of the ER and, thus, CK movement into the cell and cellular compartments, could be important for CK signaling and remains to be elucidated.

2.3.2 Histidine phosphotransferases act as cytoplasmic nuclear shuttles

Following the His-Asp phosphorelay, the signal is transmitted from receptors to the nucleus via phosphotransfer proteins. The first link between CK and TCS downstream of the receptors was provided by the interaction of *Arabidopsis* HP (AHP2) with CRE1 in *Escherichia coli* that activated bacterial RRs[232].

The predicted role of AHPs as hub between CK receptors and RRs was confirmed *in vitro* by the AHP interactions with the majority of the other TCS proteins[233,234] and *in vivo* by transient expression experiments, showing that fusions between the green fluorescent protein (GFP) and two *Arabidopsis* HPs (AHP1 and AHP2) were translocated from the cytoplasm to the nucleus[218,235]. The *Arabidopsis* genome encodes five HP proteins (AHP1 to AHP5) that

carry the conserved phospho-accepting His residue and function as positive regulators of CK signaling in a redundant manner[236]. Their phosphostransfer activity has been demonstrated *in vitro*[237–239]. A pseudo-HP protein, AHP6, lacks the conserved histidine residue and negatively regulates CK signaling because it interferes with the phosphotransfer[240].

The *ahp1*, *aph2*, *ahp1*, *aph3*, *aph4*, and *aph5* mutants display a phenotype similar to that of *ahk2*, *ahk3*, and *ahk4* during vegetative growth, but to that of *cki1* mutants during female gametophyte development, suggesting that, whereas CKI1-AHP-dependent signaling is essential for megagametogenesis, CK receptor-AHP–mediated signaling mainly regulates vegetative growth[241]. The negative role of AHP6 in CK signaling was demonstrated in protoxylem formation[240] and LR development[242].

2.3.3 Mediating the transcriptional response to CK via RRs

The final components of the plant TCS are the RRs that translate the signal into CK-regulated growth and development. Sequence analysis of the *Arabidopsis* genome revealed 23 response regulators (ARRs)[243,244]. Based on the phylogenetic analysis of their amino acid sequences and transcriptional induction by CKs, the typical ARRs have been classified into two classes, A-type and B-type ARRs[245,246], but additional phylogenic analysis of all RRs that contain a receiver domain has divided the RR proteins into four different groups: A-type, B-type, C-type, and pseudo RRs (PRRs) (Fig6A-D)[247–250].

The B-type ARR family consists of 11 members (ARR1, ARR2, ARR10 to ARR14, and ARR18 to ARR21) and contain a receiver domain at the N-terminus, a GARP (after maize GOLDEN2, **A**RR B-class from *Arabidopsis*, and *Chlamydomonas* Psr1) family Myb-like DNA-binding domain at the C terminus (Fig 6B), a glutamine-rich transactivation domain, and a nuclear localization signal[237,251]. The optimal DNA motif recognized by ARR1 and ARR2 for binding is 5'-AGATT-3'[251], whereas ARR11 binds the sequence 5'-GGATT-3' *in vitro*[237]. Phosphorylated type-B ARRs act as positive regulators of CK signaling, by inducing the transcription of a subset of CK-responsive genes[250,252–254]. ARR1, ARR2, and ARR10 can also activate transcription of several type-A RRs even in the absence of CK[218,254], in accordance with a strong reduction in CK induction of multiple type-A ARR transcripts in the *arr1,arr10,arr12* triple mutants[255]. The expression patterns of the type-B ARRs are organ and tissue specific, but overlap during different developmental stages. For instance, *ARR1*, *ARR2*,

ARR10, and *AR12* are strongly expressed close to the root tips and expression patterns of *ARR2*, *ARR10*, and *ARR12* overlap in LRs[214].

Consistent with the observation that B-type ARRs positively regulate the CK signaling, *arr1,arr10,arr12* triple mutants and *arr10,arr12* double mutants display a largely reduced CK sensitivity[255–257] and plants overexpressing *ARR1*, *ARR2*[218,252,258] or with constitutively active *ARR1* and *ARR11* lacking their signal receiver domain[254] mimic plants oversensitive to CK.

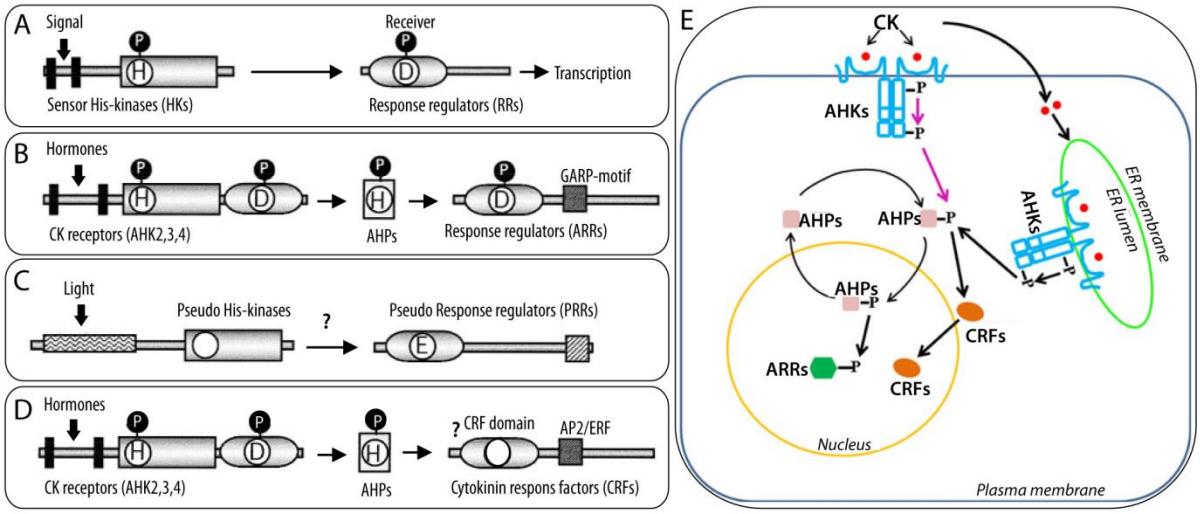

Figure 6. Two-component transducers and signaling. A. The typical TCS, consisting of HK, phospho-donor and RR phospho-acceptor in response to certain stimuli, present in bacteria and many eukaryotes, except vertebrates. **B.** Specialized His-to-Aps phosphorelay through histidine transfer proteins in response to the plant hormone CK in *Arabidopsis*. **C.** The light signal is transmitted from pseudo-HK to PRR that contains a glutamate residue in its phospho-accepting domain. **D.** Schematic structure of CYTOKININ RESPONSE FACTORS (CRFs)-new downstream components of TCS. CRF proteins interact with AHPs through a specific CRF domain by unknown mechanisms. **E**. Model of CK-mediated TCS in *Arabidopsis*. Cytokinin binding to the AHK receptor localized in the ER lumen or at the PM, resulting in the autophosphorylation of the receptor; subsequently, the phosphoryl group is transferred through the AHP and the ARR to the nucleus. Phosphorylated AHPs can also induce translocation of CRFs into the nucleus, where they, together with ARRs mediate CK responses (adapted and modified from[248,259]).

In contrast to type-B ARRs, the A-type ARRs contain only one conserved receiver domain and a short C-terminal extension instead of a DNA-binding domain[247] and function as negative regulators of CK signaling[257,260]. The 10 members of type-A ARRs (*ARR3* to *ARR9* and *ARR15*, *ARR16*, and *ARR17*) are the primary CK signaling response genes because they are rapidly transcriptionally up-regulated by exogenous CKs[261]. Type-A ARRs can be activated directly via TCS through the HKs and HPs, but are also under the control of type-B ARRs as their direct transcriptional targets[218,254]. Furthermore, type-A ARRs can down-regulate the primary CK response via a negative feedback loop[218], can act as positive or negative regulators of downstream CK responses[218,262], or modulate other signaling pathways

through protein-protein interactions[263]. Their subcellular localization is diverse and can be detected in the nucleus, cytoplasm, or both[211,218,252] . The expression of type-A ARRs is the highest in roots, but is also detectable in other plant organs as well[209,257]. Together with considerable overlap in their expression patterns and weak phenotypes of single loss-of-function mutants, type-A ARRs might be functionally redundant[257].

Similar to type-A ARRs, type-C ARRs (ARR22 and ARR24) possess only a phospho-accepting receiver domain and any DNA-binding domain[247]. However, the sequence similarity of this domain with other RRs is weak and appears to be more related to the receiver domain found in the hybrid HK receptors[258,264]. Moreover, in contrast to type-A ARRs, type-C RRs are not induced by CK[258]. ARR22 is localized in the cytoplasm, *ARR22*::*GUS* accumulates specifically in the chalaza of developing seeds, and its expression can be up-regulated at this location by wounding[258,265]. The transgenic line overexpressing *ARR22* displayed a CK-associated dwarf phenotype that is very similar to that of the *wol* mutant[258].

Spatial expression analysis has revealed that the *ARR24*::*GUS* expression is primarily restricted to pollen grains. Although the purified proteins have the ability to undergo phosphorylation *in vitro* and their gene expression is associated with reproductive tissues[258], lack of phenotypes in loss-of-function mutants[265] and of sequences distinctive from the other ARRs suggest that they may be involved in response to other signals. The role of these genes *in planta* remains to be uncovered.

By sequence analysis of RRs, five additional pseudo RRs (PRRs) have been identified in *Arabidopsis*. PRRs contain a pseudoreceiver domain that is similar to the receiver domain of a two-component response regulator, but the key Asp residue that accepts a phosphoryl group from a sensor kinase is changed to glutamate[266]. Similar to type-B ARRs, PRRs also have a C-terminal extension DNA-binding domain, but, instead of a Myb-like motif, a CCT plant-specific motif (*CONSTANS*, *CONSTANS-like* and *TIMING OF CAB 1*) is present (Fig. 6C)[266,267]. The small family of PRRs consists of five members (PRR1, PRR3, PRR5, PRR7, and PRR9)[267] that were implicated in circadian rhythms and other light responses[268]. Although PRRs have not been thought to be involved in CK signaling, three of five PRRs were transcriptionally regulated by this hormone[269]. As a role for CKs in circadian rhythms had been demonstrated[270], it remains to be elucidated whether the transcriptional regulation of PRRs is linked to TCS and, as such, contributes to the CK modulation of light-dependent processes.

2.3.4 New components of CK responses

Traditional components of TCS directly downstream of the receptors and AHPs are usually limited to the RRs mentioned above. Recently, a novel class of RRs has been identified as a side branch of the CK signaling pathway downstream of the receptors and phosphotransfer proteins[271–273]. The CYTOKININ RESPONSE FACTOR (CRF) family comprises of twelve members (CRF1 to CRF12) and is a subgroup of the APETALA2 /ETHYLENE-RESPONSE FACTOR (AP2/ERF) transcription factor family[216,274,275]. The unique CRF domain in the N-terminal region of CRF proteins was shown to be required for homodimerization and heterodimerization of these proteins and for their interaction with TCS components[271].

Phylogenetic analysis has revealed that CRFs are, similarly to ARRs, broadly present across all plant species with a large expansion in angiosperms[275]. The expression of *CRF2*, *CRF5*, and *CRF6* is transcriptionally up-regulated by CKs that require functional type-B ARRs[272]. By means of yeast two-hybrid and *in vivo* bimolecular fluorescence complementation, the direct interaction between CRFs and AHPs could be demonstrated, but no interactions with AHKs were detected[271]. This observation is similar to the interaction pattern of type A and type-B ARRs that interact with AHPs and not with AHKs[233]. Specific CRF-RR interactions were found only for ARR10 and CRF6 and both CRF1 and CRF2 with ARR7 and ARR12[271], suggesting that additional interactions between downstream components could eventually modulate CK responses.

By microarray analysis, CRFs and type-B ARRs were shown to transcriptionally activate many common target genes and each of these transcription factor groups to regulate distinct targets[272]. Overlapping expression patterns of *CRF* genes were detected with promoter reporter GUS or GFP in embryos and in *Arabidopsis* seedlings, including roots, LRP, shoots, and vascular tissues of various aerial organs[216] (Chapter 4), hinting at a redundant function for CRFs. Consistently with their expression patterns, *CRF* genes are involved in the development of embryos, LRs, cotyledons, leaves, and vascular patterning[216,272] (Chapter 4).

In accordance with a role of *CRF* genes in CK signaling, phenotypes of *CRF* loss-of function mutants and overexpressing transgenic lines revealed impaired CK responses in terms of root length, root meristem size and LR initiation (Chapter 4). However, the embryo-lethal phenotype of the *crf5*,*crf6* double mutant[272], which is distinct from the CK receptor

triple mutant, suggests that *CRF* genes may respond to other signals as well. Indeed, a recent report showed the induction of *CRF6* expression by abiotic stresses[273]. In addition, CRF6 negatively regulates leaf senescence and the CK-mediated induction of CRF6 in leaves is largely reduced in the *ahk3* mutant, providing further evidence for CRF6 being a transcriptional target of the CK signaling pathway downstream of this receptor[273].

These functional, genetic and biological studies on CRF introduced a new branching point into the CK signaling, in which CRFs are placed downstream of AHKs and AHPs and regulate, in parallel with type–B ARRs, common and distinct target genes thus translating the CK signal into various aspects of plant development. This result also provides the baseline for future studies on the role of CRFs in specific processes throughout the plant life.

In conclusion, characterization of CK metabolism and signaling during recent years has led to the identification of key genes involved in the regulation of developmental changes in response to CKs. The following section will focus on the link with the regulatory pathways that respond to internal signals and together with auxin govern plant embryogenesis as well as postembryonic development.

3 Role of CK in plant development underlined by crosstalk with auxin

Cytokinin is a plant hormone that plays positive and negative regulatory roles in many aspects of plant growth and development. It stimulates the formation and activity of shoot meristems, retards leaf senescence, inhibits root growth and branching, and plays a role in development of reproductive organs and in seed germination as well as in responses to the environmental cues, such as light, nutrients, pathogens, and biotic and abiotic stresses (reviewed in[217]). Moreover, CK has been shown to interact with the plant hormone auxin in an antagonistic manner to mediate various developmental responses. The antagonistic role of auxin and CK has arisen from *in vitro* tissue culture experiments[276]. High CK/auxin ratios led to shoot regeneration, whereas low ratios promoted root formation. These interactions occur at multiple levels, including metabolism, transport, and signaling (reviewed in[277]) and are crucial for many aspect of plant development.

3.1 CK signaling and crosstalk with auxin in early embryogenesis

Until recently, the role of CK signaling during embryo development was unclear because the triple CK receptor mutant did not display any embryonic defect[175]. However, the requirement of CK signaling for root stem-cell niche specification had been demonstrated[278]. By means of a synthetic TCS reporter, the first CK signaling could be shown in the hypophysis of the 16-cell stage embryo. After the asymmetric division, the CK activity is repressed by ARR7 and ARR15 in the basal cell and retained in the apical daughter cell, suggesting that CK signaling is required to govern correct divisions needed to establish the stem cell niche, also known as QC (Fig. 7). During the heart stage, *AHK4*, *AHP2*, *AHP3*, *AHP5*, and type-A and type-B *ARR* genes were expressed[278]. Moreover, expression of the type-A *ARR7* and *ARR15* was induced, not only by CK, but also by auxin (Fig. 8A-E) that, in turn, repressed the CK signaling in the basal cell, allowing the formation of the root apical meristem (RAM) (Fig. 8F)[278].

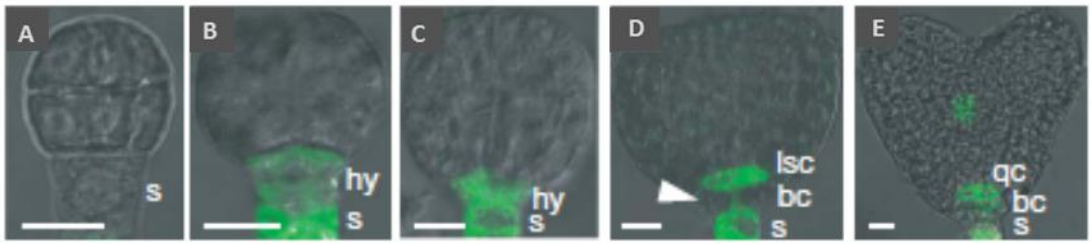

Figure 7. TCS signaling throughout the early stages of embryogenesis labeled by GFP-tagged TCS protein. A. Eight-cell stage (no expression) **B.** Sixteen-cell stage with TCS in the hypophysis (hy). **C-D.** Transition to the basal daughter cell in **D** with decreased TCS. **E.** Heart stage embryo with TCS at the SAM (adapted from[278]).

Asymmetric auxin distribution plays a crucial role in the specification of RAM during embryogenesis and auxin signaling is actively present in the basal embryo cell (Fig. 8E)[109,115]. The auxin action in the establishment of an apical-basal axis during early embryogenesis is mediated by several key players, including PIN proteins, the ARF5/MP transcription factor and its inhibitor IAA12/BDL[115,279]. Screening for transcriptional targets of MP identified the *CRF2*-encoding gene *TARGET OF MONOPTEROS 3* (*TMO3*)[280], a downstream CK signaling component[272]. CRF2 also regulate transcriptionally *PIN1* and *PIN7* by binding to their promoter regions and the loss-of-function *crf2* mutant displays embryo defects (Chapter 4), supporting the role of CRF2 as a downstream link between auxin and CK pathways in early embryogenesis (Fig. 8G). The transient and antagonistic interactions between auxin and CK

responses during early embryogenesis[278] suggest a comprehensive crosstalk between these two hormones during organogenesis.

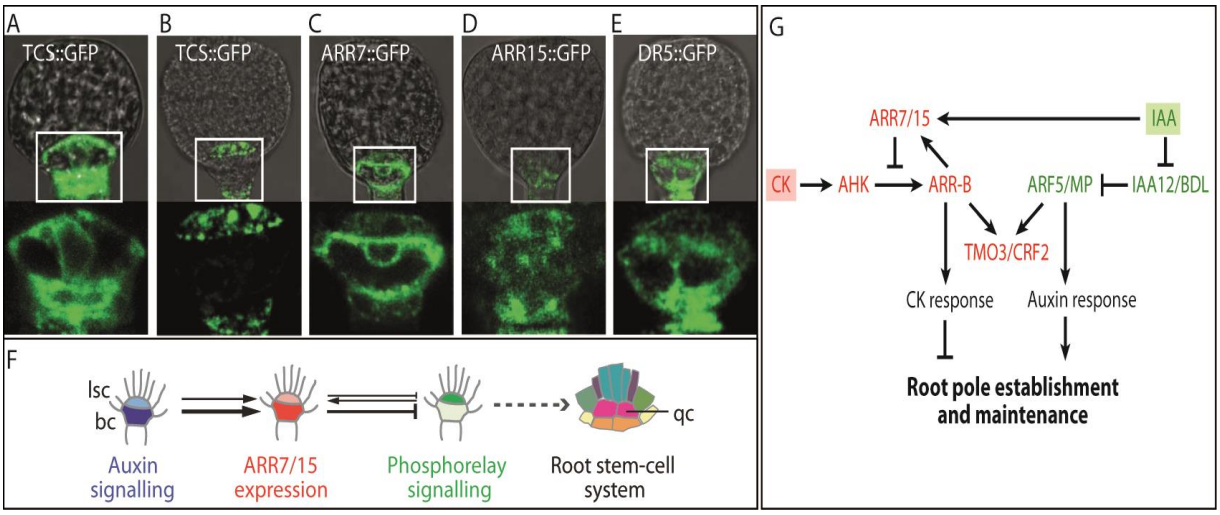

Figure 8. Auxin-CK crosstalk during early embryogenesis. A-D, *TCS*, *ARR7*, *ARR15*, and *DR5* expression during the basal daughter cell stage**. A,B** TCS in the hypophysis. **C,D E**xpression of *ARR7* and *ARR15* in the basal cell. **E** Auxin expression in basal cell. **F** Model of auxin-mediated down-regulation of CK signaling important for the establishment of root stem cell niche. **G** Complex network of genes involved in auxin-CK interplay during this process (adapted from[278,281]).

3.2 The negative role of CK in root development is counteracted by auxin

After the root QC is established during embryogenesis, CK-mediated TCS has been found to play a role in postembryonic root growth and development. Balanced root growth is maintained by control between cell division, differentiation, and elongation rates that are confined to morphologically distinct zones, such as the meristematic, transition, elongation, and differentiation zones[282].

Complete absence of CK perception reduces the meristem size and root length[175,229] and primary root growth is diminished in *ahk2,ahk3,ahk4* triple and *ahp1,ahp2,ahp3,ahp4,ahp5* quintuple mutants and arrested in the *arr1,arr10,arr12* triple mutant[175,213,229,236,283]. Furthermore, exogenous CKs reduce the number of meristematic cells, resulting in a decrease in meristem size and primary root growth, whereas mutants with reduced biosynthesis, such as the *ipt3,ipt5,ipt7* triple mutant, and plants with ectopic *CKX* expression or the TCS mutant plants *ahk3* and *arr1,arr12,* produce a large RAM[284]. The enlarged root of the CK receptor mutants *ahk3* and *ahk4*, in contrast to the reduced root length of the CK receptor triple mutant, suggests that basal levels of CK are crucial for normal RAM growth and development. Studies on LOG proteins, which are responsible for the production of active CK

species, revealed that the primary root growth of *log* multiple mutants containing *log3,log4,log7* is cumulatively inhibited and, finally, terminated in the septuple mutant[180], confirming the requirement of minimal threshold levels of active CKs for normal root growth.

Based on the expression of the *AHK3* in the transition zone and the root meristem phenotype of *ahk3,arr1* and *arr12* that phenocopies CK biosynthesis mutants, CK-mediated TCS was found to act at the transition zone to control the cell differentiation rate[284]. However, the control of the root meristem stem cell number and proliferation requires the interaction with auxin[67].

In contrast to the negative role of exogenous CKs on root growth[284], low concentrations of auxin enhance primary root growth[285]. Two major regulators of stem cell formation and maintenance *WUSCHEL RELATED HOMEOBOX5* (*WOX5*), expressed in the QC and the auxin-responsive *PLT* genes, expressed in the stem cells surrounding the QC, act downstream of the auxin signaling mediated by IAA17/AUXIN RESISTANT3 (AXR3), ARF10, and ARF16 signaling components[286].

A role for the auxin-CK crosstalk in controlling meristem size and overall root growth emerged from the characterization of the *SHORT HYPOCOTYL 2* (*SHY2/IAA3*) gene that negatively regulates auxin transport by decreasing the *PIN* expression (Fig. 9A). SHY2 is a direct transcriptional target of B-type ARR1 and ARR12 in response to CK signals through the AHK3 receptor[67]. CK signaling also alters the expression of the PIN efflux carriers via the type-A RRs[73], whereas, auxin, in turn, mediates the SHY2 degradation to control the PIN-mediated cell-to-cell auxin transport and redistribution, important for cell expansion in the RAM[287]. SHY2 is also part of a negative feedback control in which a reduced CK level is achieved via down-regulation of the CK biosynthesis gene *IPT5*[67]. The cell proliferation regulator BREVIS RADIX has been shown to control cell differentiation together with SHY2 in an antagonistic manner through a complex network assembling on the *PIN3* gene[288]. Recently, DA1-RELATED PROTEIN2 (DAR2) has been uncovered as a new regulator involved in controlling root meristem size. DAR2 acts downstream of CK and SHY2 to maintain a normal auxin distribution by influencing the auxin transport[289]. Loss-of-function *dar2* mutants produce small root meristems and show decreased cell division and early cell differentiation due to a reduced stem cell niche activity in the root meristem. *DAR2* expression is restricted to the border between the transition zone and the elongation zone[289], overlapping with the *AHK3* receptor expression[67], further implying a connection between CK signaling and its role in controlling the root meristem size. *DAR2* acts upstream of PLT1/PLT2, a transcription factor regulated by *PIN* genes through a feedback loop

mechanism, affecting root stem cell niche activity and thus root meristem size. Therefore, the identification of *DAR2* provides a novel link between several key regulators influencing root meristem size[289].

LRP formation and LR elongation are important processes that regulate the root architecture, of which the mounding capacity facilitates the plant to adapt to environmental changes. LR formation is initiated from pericycle FCs adjacent to the xylem pole that acts as extended meristems and triggers LRP development at discrete locations[116,137,290–292]. LR development involves several stages: initiation, primordium formation, emergence, own meristem establishment, and elongation[137,291].

The application of exogenous CK has also been shown to inhibit LR formation[293,294]. Analysis of various transgenic lines with altered CK levels have confirmed that CK plays a negative role in LR formation[167,168,255,257,295–297]. By means of the B-type cyclin fused to GUS (*CYCB1::GUS*) as cell division marker, CK specifically affected the first anticlinal divisions of pericycle cell in the FCs of the LRs[293,294]. Besides FCs, also young primordia are sensitive to CK and their development is arrested, whereas once new LR meristems are established, the later developmental stages are not altered by CK and LRs are able to elongate[293]. In other words, the CK action in the LR development is restricted to the initiation and primordium formation stages before LR emergence.

Analysis of mutants with reduced CK signaling revealed that lack of AHK2 and/or AHK3 enhanced LR formation. In addition, an increased number of LRs after CK treatment when the AHK4/CRE1 receptor was knocked out[67,229,294,297,298], indicates that depending on the CK levels and developmental stage, different CK receptors may be involved in repression of LR initiation and formation.

As establishment and development of LRs is under the control of auxin and CK, a crosstalk between these pathways has been suggested[293,295]. Cytokinin-auxin crosstalk has been reported in LR development at the transcriptional and also post-transcriptional level[72,293]. Cytokinin alters the transcription of *PIN1*, *PIN3*, and *PIN7* that affect PIN-dependent LR initiation[293]. PIN1 and PIN7 have been shown to be direct targets of *CRF* transcription factors, whose overexpressing or loss-of-function mutants exhibit altered LRP initiation and development (Chapter 4). Elevated transcript levels of several transcription factors from the NO APICAL MERISTEM (NAM)-ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF)-CUC (acronym NAC) family (including NAC1) in *ipt1,ipt3,ipt5,ipt7* CK-biosynthesis multiple backgrounds have been detected by microarray analyses[299]. *NAC1* is

induced by auxin and mediates auxin signaling to promote LR development. *NAC1* overexpression can restore LR formation in the auxin response mutant *tir1*, indicating that NAC1 acts downstream of TIR1[300] . Another work suggested that up-regulation of several NAC proteins leads to enhanced LR development of CK-deficient plants, an effect which might be mediated by auxin[299]. Hence, NAC TFs could be the integrative factors between auxin and cytokinin signaling pathways.

Post-transcriptional regulation of LR organogenesis occurs via CK-signaling, perceived by AHK4-ARR2 and 12 components, which promotes the PIN1 targeting to the vacuoles. This leads to an arrest of the early LRP stages indicating that a certain concentration of auxin is required to promote LR organogenesis[72].

Recent observations demonstrated that CK and auxin control primary root development and LR organogenesis in an opposite manner by interacting with each other at multiple levels.

3.3 CK controls shoot development in a positive fashion via interaction with a local transcriptional network and auxin

Like root growth, shoot growth is provided by the apical meristems, where cell divisions mostly occur. The SAM is a small group of pluripotent stem cells that generate all of the aerial parts of the plant. The balance between cell maintenance, proliferation activity, and differentiation in the SAM is regulated by the coordinated action of several transcription factors and molecular signals, including CK and its crosstalk with auxin (Fig. 9B)[301,302]. Several studies have provided the evidence that CK plays a positive role in the regulation of the SAM size and activity[167,168,175,303]. For instance, the *ahk* triple receptor mutant displayed an elevated SAM activity leading to a reduced SAM size with fewer cell layers and fewer cells per layer[175]. Similarly, a decreased SAM size has been observed in transgenic plants with low levels of CK by reducing the CK biosynthesis through knocking-out of *IPT* genes[177] or enhancing CK degradation by overexpressing *CKX* genes[168]. An enlarged SAM size was found in the *ckx3,ckx5* double mutant[304], further supporting a positive role for CK in SAM proliferation and development.

In concert with CK, a local transcriptional network regulates the SAM maintenance and function. The transcription factor WUSCHEL (WUS) defines the central zone that is functionally equivalent of the QC in the RAM and determines the number of overlying pluripotent stem cells. A negative feedback loop via the CLAVATA (CLV) signaling pathway represses the *WUS* expression and thus controls the number of *WUS*-expressing cells and the

maintenance of the stem cell numbers[305]. CK has been shown to up-regulate the *WUS* expression through an AHK2/AHK4-dependent pathway[157]. The expression pattern of *WUS* and *AHK4* overlap in the SAM, suggesting that CK may play a role in root meristem patterning within the SAM[157]. In turn, *WUS* enhances the CK perception by repressing *ARR5, ARR7*, and *ARR15*, members of the type-A ARR family that negatively regulate the CK signaling[157,306]. Moreover, ARR7 and ARR15 are also essential for expression of CLV3 receptor kinase[302]. In addition to WUS, the expression of *ARR7* and *ARR15* is repressed via direct binding of the auxin response factor MP/ARF5 to their promoters[302]. The Aux/IAA–ARF5/MP signaling pathway is induced by local auxin accumulation produced by *YUC* genes (Fig. 9B)[307].

In contrast to the positive effect of WUS on CK signaling, the negative feedback loop between *WUS* and the CK biosynthesis gene *LOG4*, expressed in the SAM epidermis, limits the positive effect of CK on cell division. Thus, apically produced CK via *LOG4*, together with the CLV signaling, plays a crucial role for patterning the WUS domain within the stem cells[308].

Maintenance of the stem cell niche is further regulated by STM, a class I KNOTTED1-like homeobox transcription factor that is expressed ubiquitously throughout the SAM and prevents stem cells from differentiation[309–311]. STM induces CK biosynthesis through transcriptional up-regulation of IPT7, enhancing the CK signaling that, in turn, triggers an increase in STM transcript levels, an indication of a positive feedback loop between STM and CK signaling[311,312]. In contrast to CK, STM is repressed by auxin at the peripheral zone of the SAM, with down-regulation of the CK-biosynthesis as a consequence[130,155].

In conclusion, the auxin-CK crosstalk together with a complex transcriptional network and multiple feedback loops (Fig. 9B) mediate the proper patterning and establishment of SAM. CK inhibits stem cell differentiation, whereas elevated auxin levels present at the margins of the shoot meristem trigger organ initiation[108,313] via rapid down-regulation of the CK biosynthesis in the shoot[314] and, hence, act in an antagonistic manner.

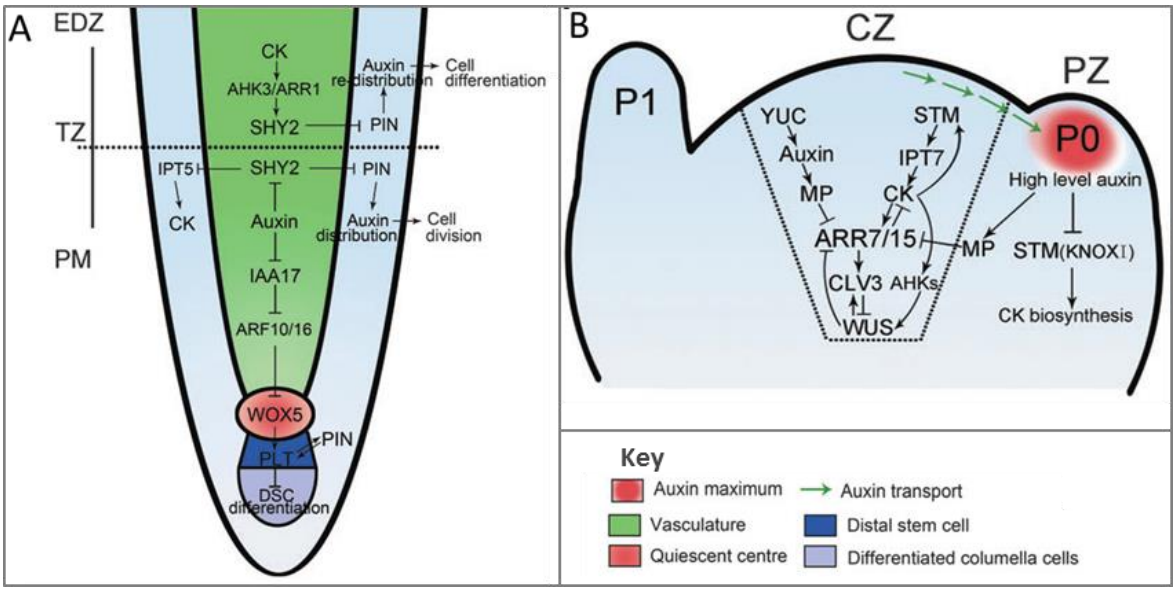

Figure 9. Auxin and CK crosstalk in the regulation of root and shoot meristem. A. In the root meristem, CK signaling via AHK3-ARR1 stimulates the expression of *SHY2*, which negatively regulates *PIN* expression and thus the auxin redistribution and cell differentiation. In turn, auxin stimulates the SHY2 degradation, allowing PIN-mediated auxin redistribution and cell division. SHY2 also decreases CK levels via down-regulation of the CK biosynthesis gene *IPT5*. Auxin signaling via IAA17/ARF10/ARF16 also plays an important role in stem cell formation and maintenance by modulating the expression of *WOX5* and *PLT*. In addition, *PLT* is regulated by *PIN* genes via a feedback loop mechanism. DSC, distal stem cell; EDZ, elongation differentiation zone; PM, proximal meristem; TZ, transition zone. **B.** Development of SAM requires auxin-mediated repression of the ARR7 and ARR15 CK signaling components. Auxin negatively regulates the expression of *ARR7* and *ARR15* via *MP*, whereas CK stimulates their expression through a *STM*-dependent pathway. In addition, *WUS*, which is important for stem cell maintenance, represses *ARR7* and *ARR15* and is up-regulated by CK. In the peripheral zone, a high level of auxin transported from the central zone inhibits the biosynthesis of CK by suppressing *STM*, which acts as an inhibitor of stem cell differentiation. CZ, central zone; P0/P1, organ primordia; PZ, peripheral zone (adapted from[315]).

In addition to normal shoot development, the auxin-CK interplay is also pivotal in *de novo* shoot regeneration. Different ratios of exogenous auxin and CK mediate various cell fates in the callus, indicating the importance of specific hormone levels and crosstalk between these two hormones during *de novo* organ regeneration. During callus formation, exogenous application of auxin leads to up-regulation of the *AHK4* expression, whereas exogenous CK regulates the expression of the PIN auxin efflux carriers and the auxin biosynthetic *YUC* genes in the callus[285,316].

In a callus regeneration assay, *CRF3* was up-regulated specifically in explants growing on auxin-containing callus-inducing media. Furthermore, overexpression of *CRF3* led to callus formation without exogenous phytohormones[317], implying a CRF-mediated link between auxin and CK signaling during callus formation.

A spatio-temporal auxin gradient, mediated by the coordinated local biosynthesis and polar transport, determines the spatial CK response during *de novo* shoot induction and regeneration[318]. This auxin-CK pattern was shown to be essential for spatial *WUS* induction, shoot meristem establishment, and subsequent shoot regeneration. The spatial auxin-CK interactions have been further defined by the negative regulation of *IPT* genes mediated by the auxin signaling component ARF3[318], indicating a complex auxin-cytokinin crosstalk during shoot meristem induction.

Over the last decades, research in the fields of CK and auxin synthesis, transport, metabolism, and signaling unraveled tight interconnections between these two hormones and improved our understanding of the auxin and CK interaction in the regulation of plant development and organogenesis. Early experiments have revealed that certain auxin-CK ratios either maintain cell proliferation or promote cell differentiation to form new organs, such as shoots or roots[160].

In the shoot meristem, CK stimulates stem cell proliferation, whereas auxin triggers lateral organ initiation by repressing the CK biosynthesis[130,155]. In contrast, in the root meristem, where auxin enhances cell division, CK inhibits the auxin signaling and thus cell proliferation[67]. During LR development, auxin transport and signaling induce an asymmetric cell division critical for LR initiation, whereas CK inhibits the LR initiation and LRP patterning by modulating the expression of PIN proteins[293]. Therefore, the antagonistic interactions between auxin and CK clearly play essential roles in the regulation of the balance between cell differentiation and proliferation and are important for stem cell maintenance and organ patterning.

In addition, the spatial and temporal expression of CK-related genes together with the asymmetrical distribution of auxin transporters and local auxin biosynthesis suggests that yet uncharacterized interactions might control diverse developmental aspects. Indeed, multiple feedback loops, additional crosstalk with other hormones as well as various environmental stimuli might add further complexity to the auxin-cytokinin network. Computational modeling in conjunction with experimental analysis might lead to the identification of novel interactions and components of this network.

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Chapter 2.

Cytokinin regulates root meristem activity via modulation of the polar auxin transport

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Cytokinin regulates root meristem activity via modulation of the polar auxin transport

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Abstract

Plant development is governed by signaling molecules called phytohormones. Typically, in certain developmental processes more than one hormone is implicated and, thus, coordination of their overlapping activities is crucial for correct plant development. However, molecular mechanisms underlying the hormonal crosstalk are only poorly understood. Multiple hormones including cytokinin and auxin have been implicated in the regulation of root development. Here we dissect the roles of cytokinin in modulating growth of the primary root. We show that the cytokinin effect on root elongation occurs through ethylene signaling whereas the cytokinin effect on the root meristem size involves ethylene-independent modulation of transport-dependent asymmetric auxin distribution. Exogenous or endogenous modification of cytokinin levels and cytokinin signaling lead to specific changes in transcription of several auxin efflux carrier genes from the PIN family having a direct impact on auxin efflux from cultured cells and on auxin distribution in the root apex. We propose a novel model for cytokinin action in regulating root growth: Cytokinin influences cell-to-cell auxin transport by modification of expression of several auxin transport components and thus modulates auxin distribution important for regulation of activity and size of the root meristem.

Introduction

Plant hormones play crucial roles in regulating plant development and the flexible shaping of the plant architecture in response to variable environmental conditions. The final developmental and physiological output of the hormonal signaling in plants is the typical result of combined actions of several hormonal pathways. However, our knowledge of the mechanisms involved in the hormonal crosstalk is still poor. In the regulation of root development, several hormonal pathways are involved, with auxin and cytokinin being the principal players. The whole process of root organogenesis, starting with the initiation of the root pole in embryos[1], positioning and formation of stem cell niche[2,3], maintenance of mitotic activity in proximal meristem[4–6], and rapid elongation and differentiation of cells leaving the root meristem[7] has been demonstrated to be controlled by auxin. In this context, the differential auxin distribution between cells is crucial[3,8,9]. The auxin gradients or local auxin maxima can be generated by auxin metabolic reactions, mainly by local auxin biosynthesis[6,10,11] and intercellular auxin transport dependent on the coordinated action of influx carriers of the AUX/LAX family[12], PIN efflux carriers[13,14], and members of the multidrug-resistant/P-glycoprotein (MDR/PGP) subfamily B of ATP- binding cassette (ABCB) proteins[15,16]. Accordingly, interference with the polar auxin transport disrupts the auxin distribution and results in dramatic patterning defects in the root meristem[2,3,9]. Besides auxin, cytokinin (CK) is also involved in root organogenesis. Increase in CK levels by exogenous application or overexpression of the bacterial ISOPENTENYLTRANS FERASE (IPT) gene[17,18] inhibits the root growth and reduces the meristem size. Accordingly, decreased endogenous CK levels via overexpression of the CYTOKININ OXIDASE/ DEHYDROGENASE (CKX) family genes have an opposite effect, i.e., enhanced meristem and root growth[19]. CK seems to play an important role already in the early events of root specification, because lack of the ARR7 and ARR15 components of the CK signaling pathway causes defects in the establishment of the root stem cell niche during embryogenesis[20]. Post-embryonically in the root meristem, CK does not seem to interfere with specification of the quiescent center (QC) and stem cell function, or with the overall division rate, but affects mainly the meristematic cell differentiation rate, resulting in shortening of the meristematic zone[4,5,21]. In addition, CK regulates elongation of cells leaving the root meristem[4]. The interaction between auxin and CKs in control of organogenesis has been known for years (the first information related to this phenomenon appeared decades

ago)[22] and crosstalk between CK and auxin signaling in control of root meristem size has been recently reported[21]. However, the molecular mechanisms underlying the mutual coordination of the auxin and cytokinin action and the possible crosstalk of their pathways in regulating root growth are poorly understood.

We reveal a unique mechanism of auxin–cytokinin interaction and show that CK regulates the cell-to-cell auxin transport by modulating transcription of several PIN auxin efflux carriers. We propose a model for regulation of the auxin–cytokinin balance that is critical for root organogenesis. By modulating the auxin transport CK might control the auxin levels in root meristem cells and, thus, the ratio between auxin and CK.

Results and Discussion

The CK Effect on the Root Meristem Does Not Interfere with Ethylene- Mediated Processes

Root growth depends on the production of new cells, their differentiation, and elongation. New cells are produced in the mitotically active meristem zone, whereas their differentiation and elongation occur in the more proximal part of the root tip. The plant hormone CK regulates the root meristem activity. Increase of CK levels either by exogenous application of CK or overexpression of ISOPENTENYLTRANSFERASE (IPT), a gene involved in CK biosynthesis, reduces the size of root meristems and overall root growth[5] (supporting information (SI) Fig. S1C). On the contrary, plants overexpressing CYTOKININ OXIDASE/DEHYDROGENASE (CKX) causing enhanced degradation of CK and have longer root meristems[19] (Fig. S1C). Because CK stimulates ethylene biosynthesis[23] and ethylene itself strongly affects root growth[24,25], we first examined which CK effects on the root growth are mediated by ethylene.

To address this issue, either CK-induced ethylene production was eliminated with the ethylene biosynthesis inhibitor 2- aminoethoxyvinylglycin (AVG)[26] or the ethylene response was prevented genetically by mutations in the ethylene signaling pathway, thus exhibiting typical ethylene-insensitive root growth[27]. Significantly, reduction of ethylene synthesis by AVG, and interference with the ethylene signaling in *etr1-3* and *ein2* mutants led to CK-insensitive cell elongation and overall root growth (data not shown and Fig. 1B and D) but did not interfere with the CK effect on the root meristem (Fig. 1 A and C).

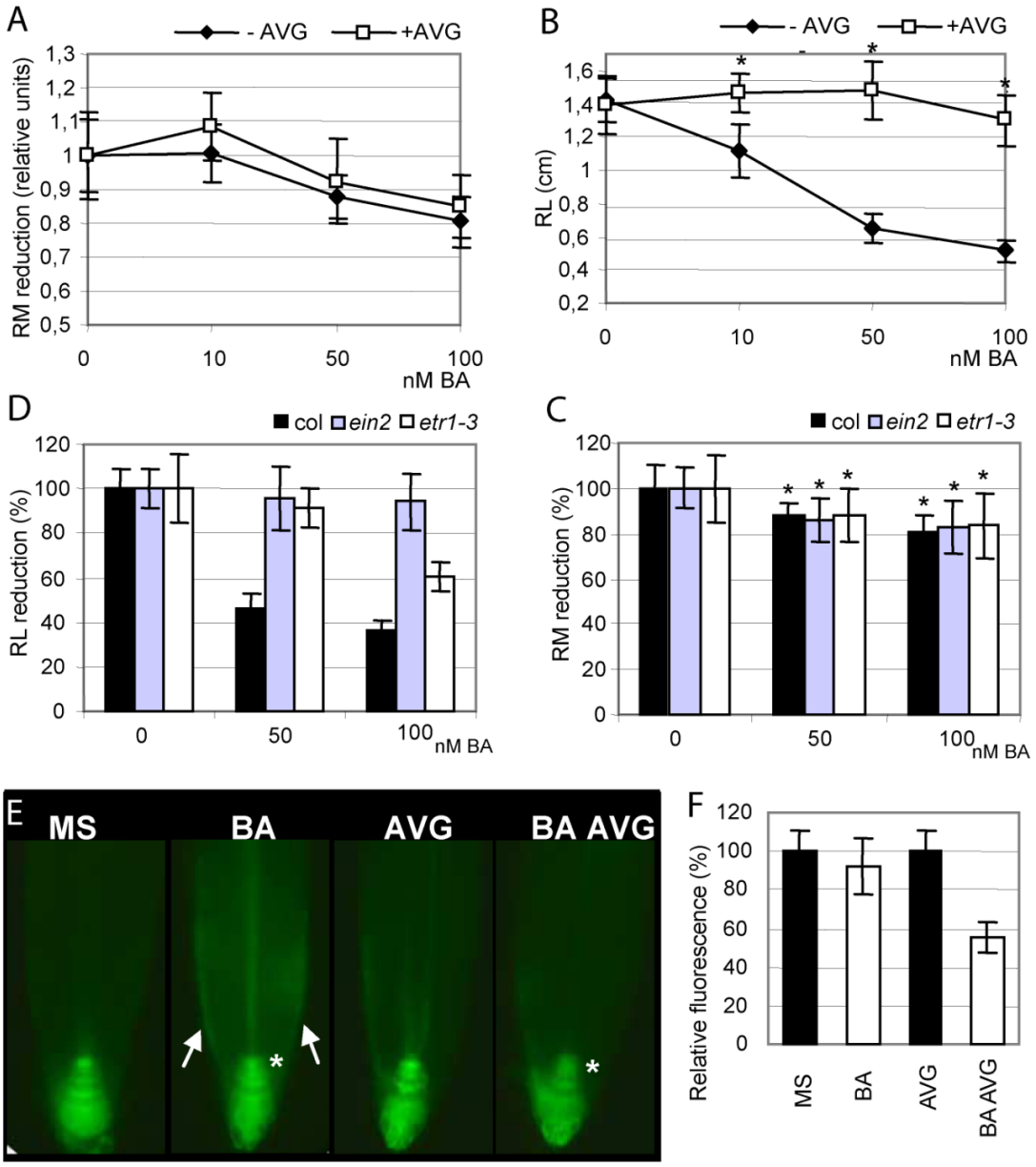

Figure 1. Ethylene-independent root meristem size modulation by CK. (A) Reduction of ethylene biosynthesis by AVG without interference with CK effect on the root meristem size (no difference between CK treated and CK+AVG-treated root meristems; Student's t test, $P < 0.05$). (B) CK-insensitive rootgrowth at reduced ethylene biosynthesis conditions (*statistically significant difference in root length between CK and CK+AVG-treated seedlings; Student's *t* test $P < 0.05$). (C) Reduced root meristem in the ethylene signaling mutants *etr1–3* and *ein2* after CK treatment (*statistically significant difference in the root meristem size between CK-treated and -nontreated seedlings; Student's *t* test, $P < 0.05$). (D) CK-resistant root growth of *etr1–3* and *ein2*.(E) CK-induced ectopic expression of *DR5::GFP* reporter in outer layers of the root meristem (lateral root cap and epidermis; arrowheads). Reduction of ethylene biosynthesis by AVG diminishes the DR5 ectopic expression. CK-reduced DR5 expression in QC (asterisks). (F) Quantification of *DR5::GFP* expression by image analysis in QC of roots treated with CK and CK+AVG; expression of *DR5::GFP* is significantly reduced after CK treatment under conditions of reduced ethylene biosynthesis (*statistically significant difference between AVG and CK+ AVG-treated roots, Student's t test, $P < 0.05$). Six-day-old seedlings grown on media containing 100 nM BA, 200 nM AVG (if not marked differently). RM, root meristem; RL, root length; MS, Murashige and Skoog medium only; CK represented by N^6-benzyladenine (BA); col, control Columbia seedlings. Error bars represent standard deviation (SD).

To analyze the CK effect on the cell size and cell division activity of the root meristem more in detail, we used the *CycB1;1::GUS*$_{DB}$ reporter, marking cells in the G2 stage of the cell cycle[28]. CK alone and at simultaneously inhibited ethylene biosynthesis dramatically reduced the zone of the *CycB1;1::GUS*$_{DB}$ expression, suggesting that the CK treatment might interfere with the balance between mitotic activity and differentiation of cells in the proximal meristematic zone (Fig. 2A, Fig. S2 B and D)[5], and further confirming that the CK effect on the root meristem does not depend on ethylene.

Spatial patterns of the auxin response based on auxin gradients are important factors in the regulating of a large number of plant developmental processes, including dividing root cells[3,9]. Using the auxin response reporters *DR5::GUS* and *DR5::GFP*[29], we observed that the CK treatment induces ectopic *DR5* activity in the outer layers of the root meristem and the elongation zone (Fig. 1E, data not shown). A similar change in the pattern of the *DR5* reporter expression has been demonstrated previously to be caused by ethylene[24,25,30]. Indeed, reduced ethylene biosynthesis by AVG completely eliminated this ectopic signal, indicating that CK up-regulates *DR5::GFP* expression in the elongation zone through ethylene (Fig. 1E). Remarkably, besides induction of the *DR5* activity in the epidermis of the root tip, we observed that CK attenuated the signal in the QC and columella cells and these effects became more obvious when the ethylene biosynthesis was inhibited by AVG (Fig. 1 E and F). In summary, our results dissected the ethylene-dependent and -independent roles of CK on root growth: CK regulates the root meristem size independently of ethylene, while its effect on the cell elongation and overall root growth is mediated through ethylene[24,25,30]. Ethylene-independent reduction of the auxin response in the QC and columella cells suggests a direct interaction between the CK and the auxin pathways, independent of ethylene.

CK and Nontransportable Auxin Show Similar Effects on the Root Meristem

The differential auxin distribution in the root meristem requires an active auxin transport[9]. Furthermore, some mutants defective in the activity or polar localization of PIN auxin efflux carriers[2,31] are dramatically reduced in the root meristem size, resembling the CK effect. To address whether the CK effect on the auxin distribution and root meristem activity is related to the auxin transport, we compared the effects of 2 different auxins: 1-naphthaleneacetic acid (NAA), well transportable by the polar transport system, and 2,4-dichlorophenoxyacetic acid (2,4-D), which tends to accumulate in cells because of its low affinity to the auxin efflux machinery[32]. Our results revealed dramatic differences between these 2 auxins. NAA

stimulated the mitotic activity in the proximal root meristem, as visualized by the *CycB1;1::GUS*$_{DB}$ reporter (Fig. 2A; Fig. S2A), resulting in an enlarged root meristem at lower (200 nM) concentrations, but reduced root growth already at 50 nM concentration (Fig. 2B). In contrast to NAA, 2,4-D treatment led to continuous reduction in the root meristem size and the root length at concentrations higher than 20 nM and reached the same degree of reduction of root growth at the concentration of 200 nM as did NAA at 800 nM (Fig. 2 A and C; Fig. S2C).

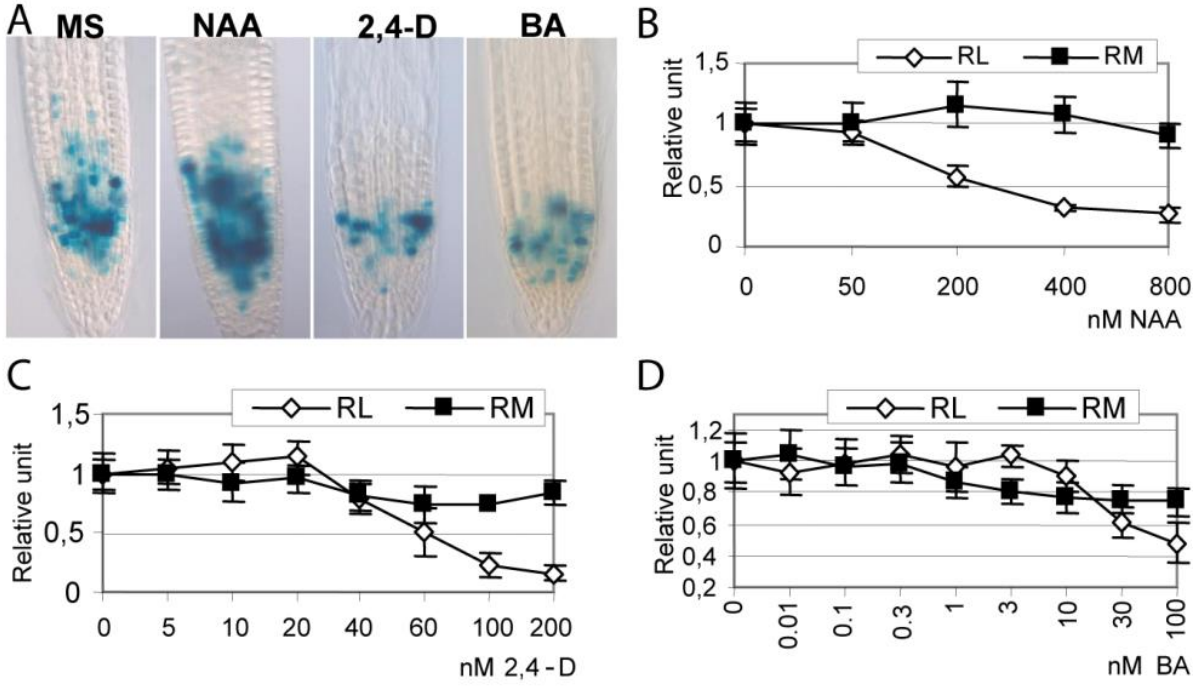

Fig. 2. CK regulation of root growth in a manner similar to 2,4-D. (A) Increased zone of CycB1;1::GUSDB reporter expression by lower (200 nM) NAA concentration. Treatment with CK (100nM BA) or 2,4-D (100 nM) reduced expression of *CycB1;1::GUS*$_{DB}$ reporter. (B) Root growth and root meristem size on NAA concentration gradients. Low NAA concentration with strong inhibitory effect on root growth still has a stimulatory effect on the root meristem size (meristems at 200 nM statistically significant, Student's t test, $P < 0.05$). (C) Gradual reduction of size of the root meristem and inhibition of root growth on 2,4-D concentration gradient (meristems at 40 nM and higher concentration statistically significant, Student's t test, $P < 0.05$). (D) Gradual reduction of the root meristem size and root growth by increasing CK (BA) concentrations (meristems at 1 nM and higher concentration statistically significant, Student's *t* test, $P < 0.05$). Six-day-old seedlings grown on media supplemented with hormones. RM, root meristem; RL, root length. Error bars represent SD.

When compared to auxins, CK mimics in many aspects the 2,4-D mode of action. It reduced the root meristem size, and it dramatically decreased root elongation already at 30 nM (Fig. 2 A and D; Fig. S2B). Moreover, low NAA concentration did not overcome CK inhibitory effect on root meristem size (Fig. S2E), suggesting that CK effect on root meristem size is not mediated exclusively by controlling overall auxin levels.

Our results demonstrate that auxins and CK modulate the root meristem size, but that they differ in concentration range at which they can act either in a stimulatory or an inhibitory mode. Importantly, the CK effect on the root meristem size resembles that of an inefficiently transported 2,4-D, hinting that the auxin transport can be a downstream target of the CK action in regulating this process.

CK Reduces Auxin Efflux in Tobacco BY-2 Suspension Cells

We tested whether CK has a direct effect on the auxin efflux—the rate- limiting process in the intercellular auxin transport. The established assay for auxin efflux quantification from cells relies on measuring the accumulation of radioactively labeled NAA ([3H]NAA) in cultured tobacco BY-2 cells[14]. In contrast to Arabidopsis cell cultures, in BY-2 culture each cell is in direct contact with the medium, so that it can render more precise quantitative data. NAA is well conveyed into the tobacco cells by passive diffusion and it is well transported out by the efflux carriers; therefore, NAA accumulation inside cells is a good measure for the auxin efflux activity[14,32]. We tested the CK effect on the auxin efflux with BY-2 cells expressing under the endogenous promoter the Arabidopsis PIN1 auxin efflux carrier tagged with RFP. In this assay, CK does not affect short term the auxin efflux (within 30 min, data not shown). However, longer CK pretreatments (24 h) led to an increase of the [3H]NAA accumulation that indicates a decrease in NAA efflux activity (Fig. 3B). Treatment with the auxin efflux inhibitor 1-naphthylphthalamic acid (NPA) has very similar, but immediate, effect on the NAA accumulation in *PIN1:GFP* expressing cell culture (ref.[16]; data not shown), further confirming that CK inhibits the auxin efflux, possibly by an indirect mechanism. We tested whether CK has an effect on *PIN* expression in BY-2 cells. Analysis of *PIN1:RFP* signal revealed that the CK treatment leads to a decreased *PIN1:RFP* expression (Fig. 3A; Fig. S1D). These data suggest that CK might modulate the auxin efflux via regulation of the *PIN* expression.

CK Modulates Expression of the Auxin Efflux Carriers of the PIN Family

The next question was whether endogenous or exogenous manipulation of CK levels in planta affects the expression of the auxin efflux carriers from the PIN family. To distinguish whether the observed changes in expression are CK-specific or related to CK-induced ethylene synthesis, all experiments were carried out in parallel in the presence of AVG as inhibitor and 1-aminocyclopropane-1-carboxylate (ACC) as precursor of the ethylene biosynthesis[26], respectively. Analyses using transcriptional and translational reporters, and real-time Q-RT PCR revealed that CK modulates the expression of several PIN genes in a manner specific for each particular auxin efflux carrier. CK negatively regulates *PIN1* expression. CK treatment strongly reduced the *PIN1:GFP* or PIN1 (visualized by antibody staining) signal in root tips (Figs. 3D and 4A; Fig. S3A). Similarly, endogenously increased CK levels, by dexamethasone- induced expression of the *IPT* gene reduced the PIN1 signal (Fig. S1A). In contrast, the decrease of CK levels by overexpression of the *CKX3* gene[19] resulted in up-regulation of the PIN1 signal (Fig. S1B). A more detailed time-lapse analysis revealed that shortly after CK application, *PIN1:GFP* expression is temporally enhanced and then gradually decreases (Fig. S3E).

However, the negative effect of CK on *PIN1* expression is complicated by the CK-induced ethylene production and subsequent induction of the PIN1 expression by ethylene[30]. Accordingly, both ACC and CK treatments up-regulated the expression of *PIN1::GUS* (Fig. S4A) and the CK-induced *PIN1* expression was completely abolished by simultaneous application of AVG (Fig. S4A). Similarly to PIN1, expression of PIN4 is also negatively controlled by CK. Expression of both PIN4::GUS and *PIN4:GFP* reporters was strongly down-regulated by CK (Fig. S4E, data not shown) counteracting the positive effect of ethylene on the *PIN4* transcription[30] (Fig. S4E). In contrast to *PIN1* and *PIN4*, the expression of *PIN2:GFP* was relatively resistant to the CK treatment and an occasionally decreased expression was observed only after longer (over 24 h) treatment with CK (Fig. 3E; Fig. S3 C and E). Because *PIN2* expression is strongly stimulated by ethylene[30], the inhibitory effect of CK was more pronounced when the ethylene biosynthesis was inhibited (Fig. S4B). In the case of *PIN3*, short-term CK treatment up-regulated and longer treatments reduced the *PIN3:GFP* expression (Fig. 3F; Fig. S3 B and E) and co-treatments with AVG and ACC did not indicate any strong influence of ethylene (Fig. S4C and S3B). In contrast to other *PIN* genes, expression of *PIN7:GFP* and *PIN7::GUS* was clearly up-regulated by CK with no significant influence of ethylene (Fig. 3G and Figs. S3 D and E and S4D). The real time Q-RT

PCR experiments in general corroborated reporter-based observation and confirmed that CK has a negative effect on transcription of *PIN1*, *PIN2*, and *PIN3* and a positive effect on *PIN7* (Fig. 3C). Nonetheless, the time dynamic and extent of response differs for each particular *PIN* gene. Higher CK concentrations (10 µM BA) even after short-term treatments show stronger effects on PIN transcription (Fig. S4F).

In summary, our results show that CK in a concentration- dependent manner differentially regulates transcription of *PIN* auxin efflux carriers in roots. Despite the fact that the effects of CK on PIN transcription are obvious and can account for CK effects on root meristem activity, there might also be additional CK-dependent posttranscriptional regulations involved.

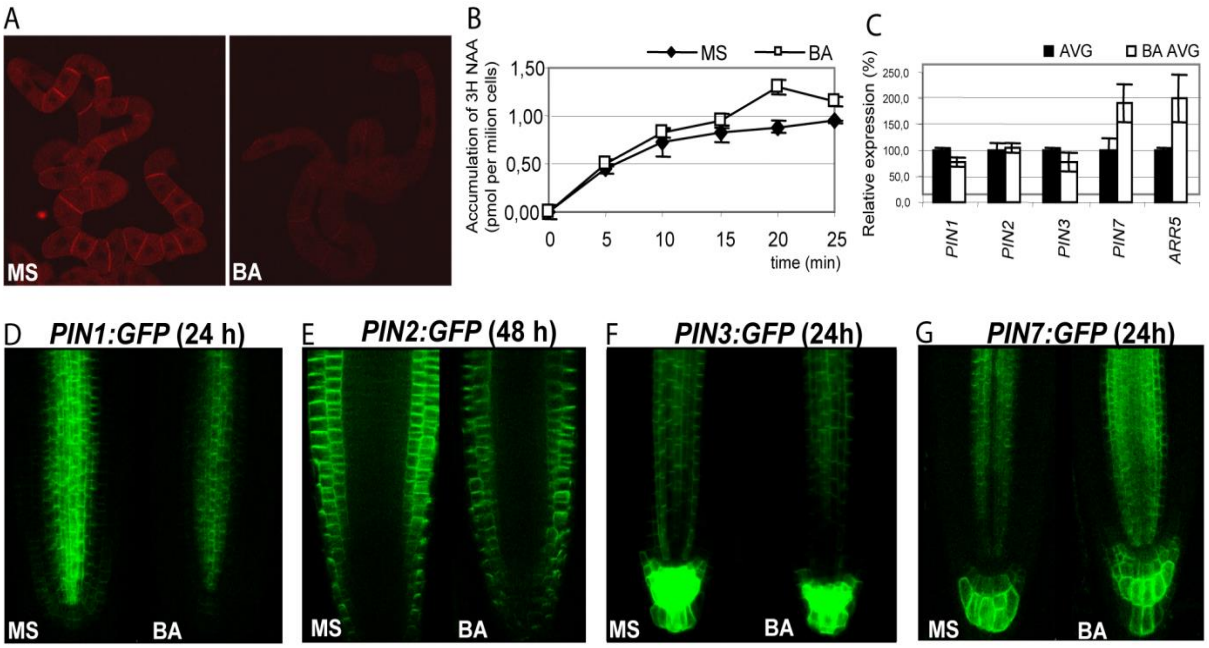

Fig. 3. Interference of CK with expression of auxin efflux carriers. (A) Reduction of *PIN1:RFP* expression by CK in tobacco BY-2 cells. (B) Increased auxin accumulation in the BY-2 cells after pretreatment with CK (5µM BA, 24 h). (C) Quantitative RT-PCR expression analysis of *PIN* genes. Six hours after CK treatment, expression of *PIN* genes is modulated. *ARR*5 used as positive control induced by CK [33]. (D) Expression of PIN1:GFP is reduced by CK treatment. (E) Down-regulation of *PIN2:GFP* expression by CK effect. (F) Down-regulation of *PIN3:GFP* expression after long-term (24 h) CK treatment. (G) Stimulation of *PIN7:GFP* expression by CK treatment. Six-day-old seedlings incubated in control or with 5µM BA supplemented media. MS, Murashige and Skoog medium only; CK represented by BA Error bars represent SD.

CK Effect on PIN1 Transcription Requires Histidine Kinase-Based CK Signaling

We investigated the requirement of the CK signaling for CK-dependent regulation of PIN transcription and root meristem growth. To corroborate whether the CK effect on the PIN1 expression does not depend on ethylene, we treated seedlings with CK and performed

immunodetection of *PIN1* in an *etr1–3* mutant that is defective in ethylene perception[27]. In this experimental setup, CK reduced the expression of PIN1 in the *etr1–3* mutant similarly to control roots (Fig. S5A), confirming that ethylene biosynthesis and perception are not required for the CK effect on root meristem growth. It is known that CK signals through the histidine kinase family of receptors[34]. To investigate the involvement of the CK perception in the CK effect on root meristem, we analyzed CK sensitivity of single and multiple receptor mutants in terms of root meristem size. While root meristems of the single CK receptor mutants *ahk2–2* and *ahk3–3* showed an almost normal CK sensitivity, *cre1–12* and its multiple mutant combinations (*cre1–12 x ahk2–2* and *cre1–12 x ahk3–3*) displayed reduced CK sensitivity (Fig. 4B). These results demonstrate that the CK control over the root meristem requires a functional CK perception pathway.

We tested whether CK receptors are similarly involved in the regulation of the *PIN1* expression. Immunolocalization of *PIN1* revealed that PIN1 expression was reduced following CK treatment of the *ahk2–2* single mutant, but exhibited increased resistance to CK and remained unchanged in *cre1–12* x *ahk2–2* double mutant (Fig. 4A; data not shown). A similar set of experiments using *PIN1:GFP* revealed that CK reduces *PIN1* expression with lower efficiency in *cre1–12* background (Fig. S5B). Thus, the CK effect on *PIN1* transcription correlates well with the CK-sensitive or CK-resistant root meristem of particular CK receptor mutant combinations. In conclusion, our data demonstrated that CK controls meristem size and that the *PIN1* expression requires functional CK, but not ethylene perception.

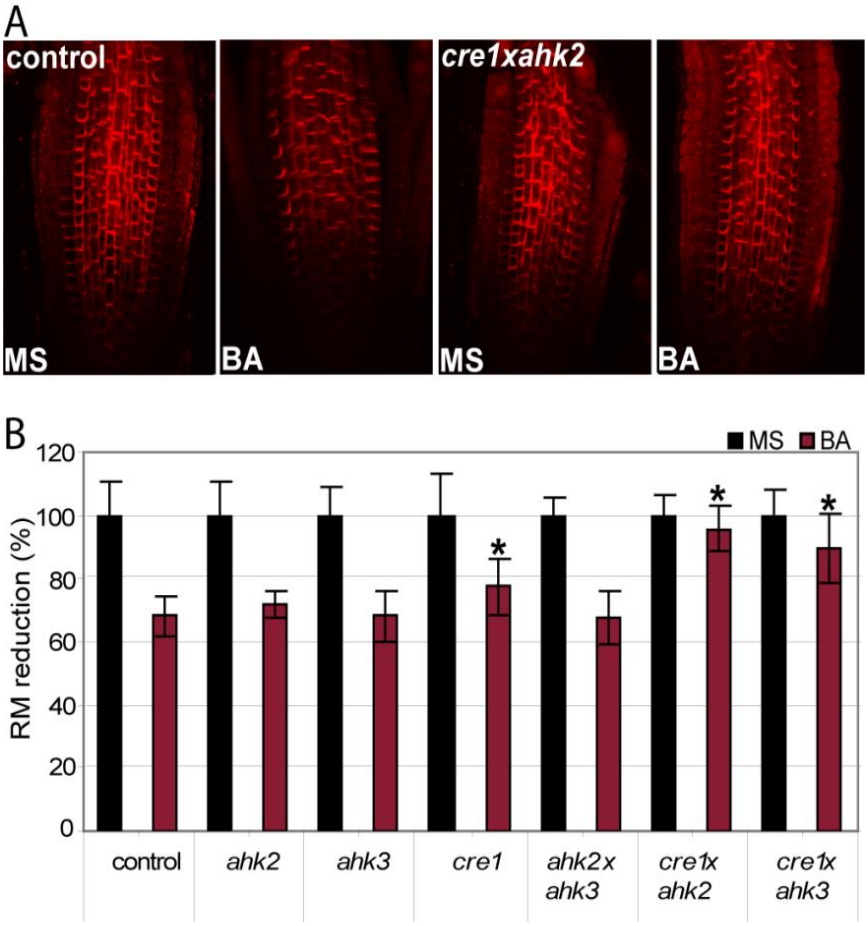

Fig. 4. Dependence of CK-mediated inhibition of PIN1 expression on CK perception. (A) Reduced expression of *PIN1* in wild-type, but not in the *cre1–12* x *ahk2–2* double-mutant root meristem after CK treatment. (B) CK-resistant root meristem of cytokinin receptor mutants *cre1–12*, *cre1–12* x *ahk2–2*, *cre1–12* x *ahk3–3*, and CK-sensitive root meristem in *ahk2–2*, *ahk3–3*, and *ahk2–2* x *ahk3–3* mutants (*significantly different from control, Student's t test $P < 0.05$). Six-day-old seedlings (A) incubated for 20 h in control media or supplemented with 1µM BA, (B) grown on control media or supplemented with 0.1 µM BA, respectively. Red signal, immunolocalization of PIN1 by PIN1-specific antibodies. Control, wild type, CK represented by BA, error bars represent SD.

Model for Auxin–CK Interaction in Root Meristems

The interaction of auxin and CK plays an important role in the regulation of plant development and organogenesis[20,35,36]. Early experiments on tobacco with tissue cell cultures revealed that auxin and CK are essential hormones for maintenance of cell proliferation, but also for the regeneration of plant organs[22]. Amazingly, the decision of whether a cell culture stays in the proliferating status or new organs, such as shoots or roots, are formed depends on the concentration ratio between these two plant hormones[22]. Another interesting aspect of the auxin–CK crosstalk is that their interaction mode strongly depends on the developmental context: they act synergistically to stimulate the mitotic activity of cells in cell cultures[22] , but

antagonistically in root and shoot branching (for review, refs.[37,38]). When focused at the apical root meristem, the role for both auxin and CK is well defined. Auxin is an important regulator of stem cell niche establishment[2,3], cell proliferation occurring in the meristematic zone[4–6], and differentiation and rapid elongation of cells leaving the meristematic zone[7]. Recently, significant progress on the role of CK has been made. CK levels have been shown to be critical for maintenance of the root meristem size and the site of CK action is primarily the regulation of the ratio between cell division and differentiation of cells leaving the meristem. In addition, a role for the CK signaling pathway has been suggested in stem cell niche establishment during embryogenesis[20]. Because auxin and CK closely overlap in the regulation of root meristems the question arises ‘‘how are their overlapping activities coordinated, and what are the molecular mechanisms behind their interaction?’’ Muller and Sheen revealed that auxin regulates transcription of *ARR7* and *ARR15*, negative regulators of the CK signaling pathway[20], suggesting that auxin might control output of the CK signaling pathway through modulation of transcription of one of its critical components.

Here, we show a novel mechanism of CK–auxin interaction involved in the control of the root meristem size. CK, by modifying the expression of several *PIN* genes, might regulate cell-to-cell auxin transport, and thus the actual level of auxin in the cells, providing the amount of signal for downstream auxin signaling.

Materials and Methods

Plant Materials and Growth Conditions

Seeds were chloral-gas sterilized, plated (0.5xMS medium with 1% sucrose), stored for 2 days at 4 °C in the dark, and then grown under a 16-h photoperiod at 20 °C. The following transgenic lines were characterized elsewhere: *35S::CKX2*, *35S::CKX* [39]; *etr1–3* and *ein2–1*[27]; *pOp::ipt/Lh6r*[40], *CycB1;1::GUS*$_{DB}$[41]; *DR5rev::GFP*; *PIN1,2,3,4,7::GUS*; *PIN1:GFP*[8] *PIN2:GFP* [42], *PIN3:GFP, PIN4:GFP, PIN7:GF*[2], *cre1–12*, *ahk2–2*, *ahk3–3* and their double-mutant combinations[43]. *PIN1::PIN1:mRFP1* gene construct was obtained from *PIN1::PIN1:GFP*[8] by replacing GFP coding sequence with mRFP1. Tobacco BY-2 cells (Nicotiana tabacum L. cv. Bright Yellow 2) were transformed by co-cultivation with Agrobacterium tumefaciens strain C58C1 carrying *PIN1::PIN1:mRFP1* according to ref.[44] and cultured as described[14]. ACC stock solution (Sigma-Aldrich) was prepared as 10 mM, AVG (Fluka) as 10 mM, 2,4-D (Duchefa) as 1 mM, dissolved with water, NAA (Duchefa) as 10 mM, dexamethasone (Sigma-Aldrich) as 5 mM, dissolved with dimethyl sulfoxide, and N6-benzyladenine (BA) (Sigma-Aldrich) as 50 mM, dissolved with 1 N sodium hydroxide. Dexamethasone induction of *IPT in pOp::ipt/Lh6r* was done as described[16,17].

Phenotypic Analysis, Microscopy and Statistics

Roots and root meristem regions were measured as published[30] with the following modification: root meristem length was assessed as the distance between the QC and the first elongating cell, the experimental image data sets were then randomly measured using the Altap Salamander Renamer (www.altap.cz) and ImageJ programs (National Institutes of Health, http://rsb.info.nih.gov/ij). Automated whole mount protein immunolocalization was done as described[45]; each sample was processed in 3 technical replicates (each ~ 10 seedlings) and all experiments were repeated at least twice. The bright field and GFP fluorescence photographs were obtained by a Zeiss Axiophot microscope equipped with an Axiocam HR CCD camera. For the confocal laser scanning microscopy, a Leica TCS SP2 AOBS was used. GFP fluorescence of membrane-localized proteins was quantified as published[30]. Images presented were processed in Adobe Photoshop. Histochemical GUS staining and root tissue clearing was done as published[45]. Data were statistically evaluated with NCSS 2007 (www.ncss.com). Equal variances of values were verified by Levene test; a Mann-Whitney nonparametric test was performed simultaneously with Student's *t* test.

PIN1::mRFP1 Expression Analysis in BY-2 Cells

For treatment with CK, stationary 7-day-old cells were inoculated into fresh medium containing (5 µM) BA after 24 h of cultivation. Cells were then incubated for another 24 h and auxin accumulation assays or confocal microscopy was performed. For observations of PIN1::PIN1:mRFP1 in BY-2 cells, a ZeissLSM5-DUO confocal microscope with a 40x C-Apochromat objective (N.A. = 1.2 W) was used.

Auxin Accumulation Assays

[3H]NAA accumulation into the cells was measured in 0.5 ml cell suspension aliquots as described[14].

Quantitative RT-PCR

RNA was extracted with the RNeasy kit (Qiagen) from root samples (last 2 mm of the root tip). DNase treatment with RQ1 RNase-Free DNase (Promega) was carried out for 30 min at 37 °C. Poly(dT) cDNA was prepared from 1µg total RNA with SuperScript III Reverse Transcriptase (Invitrogen) and quantified with a LightCycler 480 (Roche) with the LightCycler 480 SYBRGREENI Master (Roche) according to the manufacturer's instructions. PCR was carried out in 384-well optical reaction plates heated for 10 min to 95 °C to activate hot start TaqDNA polymerase (Roche), followed by 40 cycles of denaturation for 60 s at 95 °C and annealing/extension for 60 s at 58 °C. Targets were quantified with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were normalized to *ACTIN2* expression levels. All RT-PCR experiments were done at least in triplicates. The statistical significance was evaluated by the *t* test. The following primers were used:

ACTIN2 (TTGACTACGAGCAGGAGATGG and ACAAACGAGGGCTGGAACAAG),
PIN1 (TACTCCGAGACCTTCCAACTACG and TCCACCGCCACCACTTCC),
PIN2 (CCTCGCCGCACTCTTTCTTTGG and CCGTACATCGCCCTAAGCAATGG),
PIN3 (GAGGGAGAAGGAAGAAAGGGAAC and TTGGCTTGTAATGTTGGCATCAG),
PIN7 (CGGCTGATATTGATA- ATGGTGTGG and GCAATGCAGCTTGAACAATGG),
ARR5 (ACACTTCTTCATTAGCATCACCG and CTCCTTCTTCAAGACATCTATCGA).

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Supplementary Figures

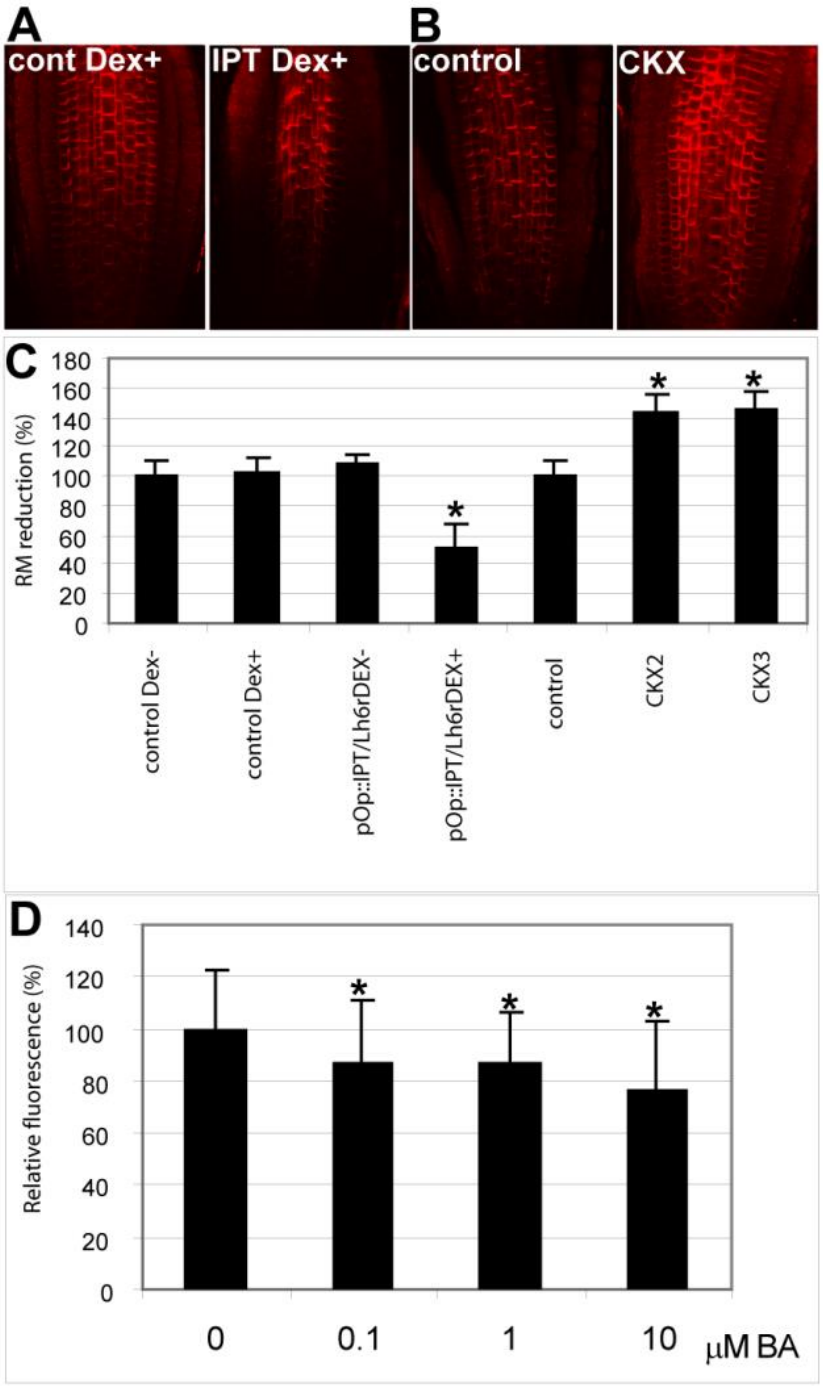

Fig. S1. *PIN1* expression affected by modulation of endogenous CK. (A) Decreased PIN1 signal caused by increase of endogenous CK after induction of IPT expression by dexamethasone in *pOp::ipt/Lh6r* line. (B) Increased PIN1 signal after reduction of CK levels in *35S::CKX3* roots. Red signal-immunolocalization of PIN1 using PIN1 specific antibodies. (C) Root meristem size in seedlings with increased CK levels after overexpression of IPT inpOp::ipt/Lh6r line and with reduced CK levels in *35S::CKX*2 and *35S::CKX3* seedlings. (D) *PIN1:RFP* signal in BY-2 cells down-regulated after CK treatment (*significantly different from control, Student's *t* test, $P < 0.05$). CK represented by BA; error bars represent SD

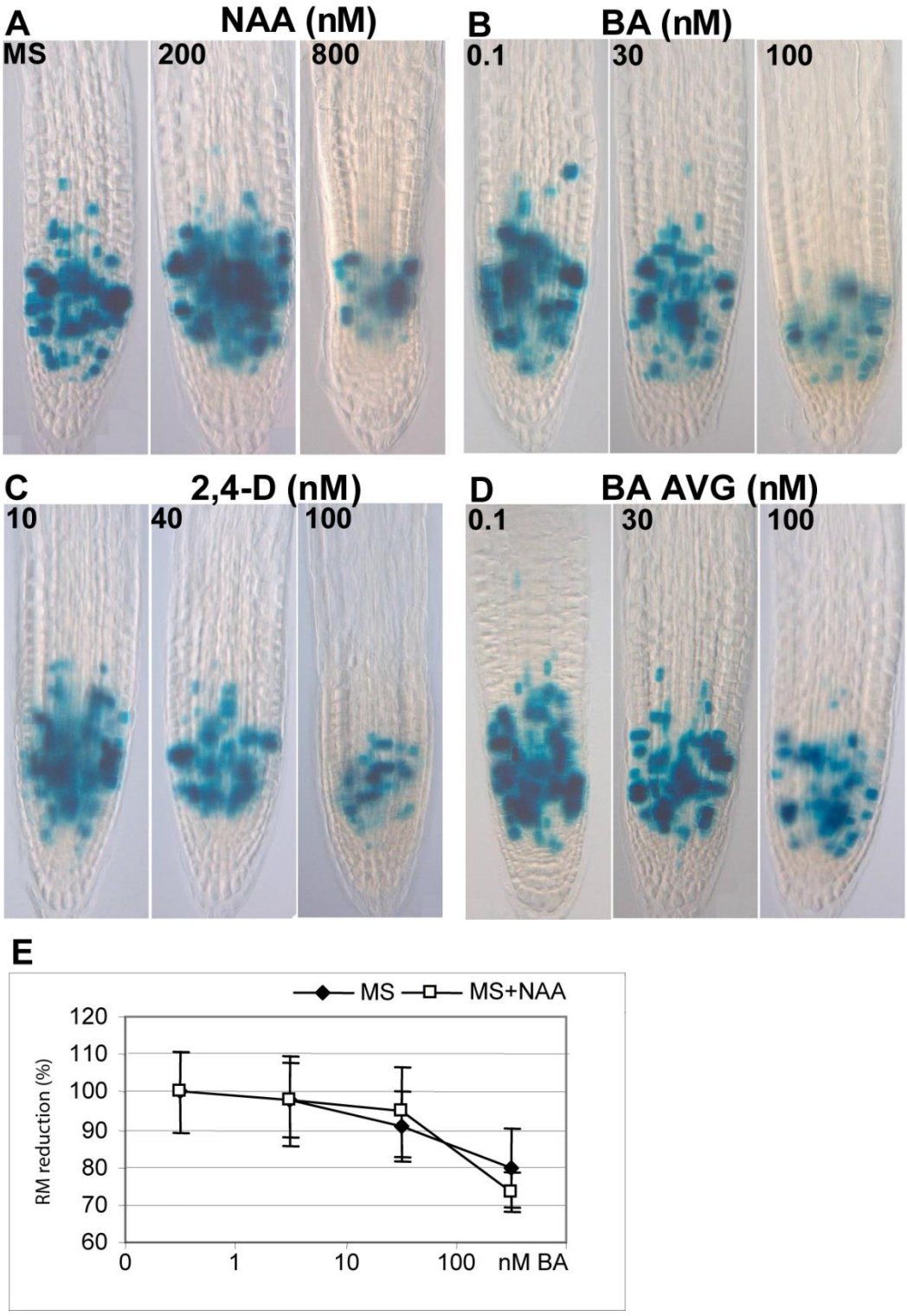

Fig. S2. Regulation of root growth by CK and auxin. (A) *CycB1;1::GUS* expression in root meristem germinated on NAA; (B) 2,4-D; (C) BA; and (D) BA at simultaneous presence of 200 nM AVG. (E) Low concentration of NAA (200 nM) does not interfere with inhibitory effect of BA on the root meristem. Seedlings germinated for 6 days on control MS medium or supplemented with hormones. MS (Murashige and Skoog) medium only; error bars represent SD.

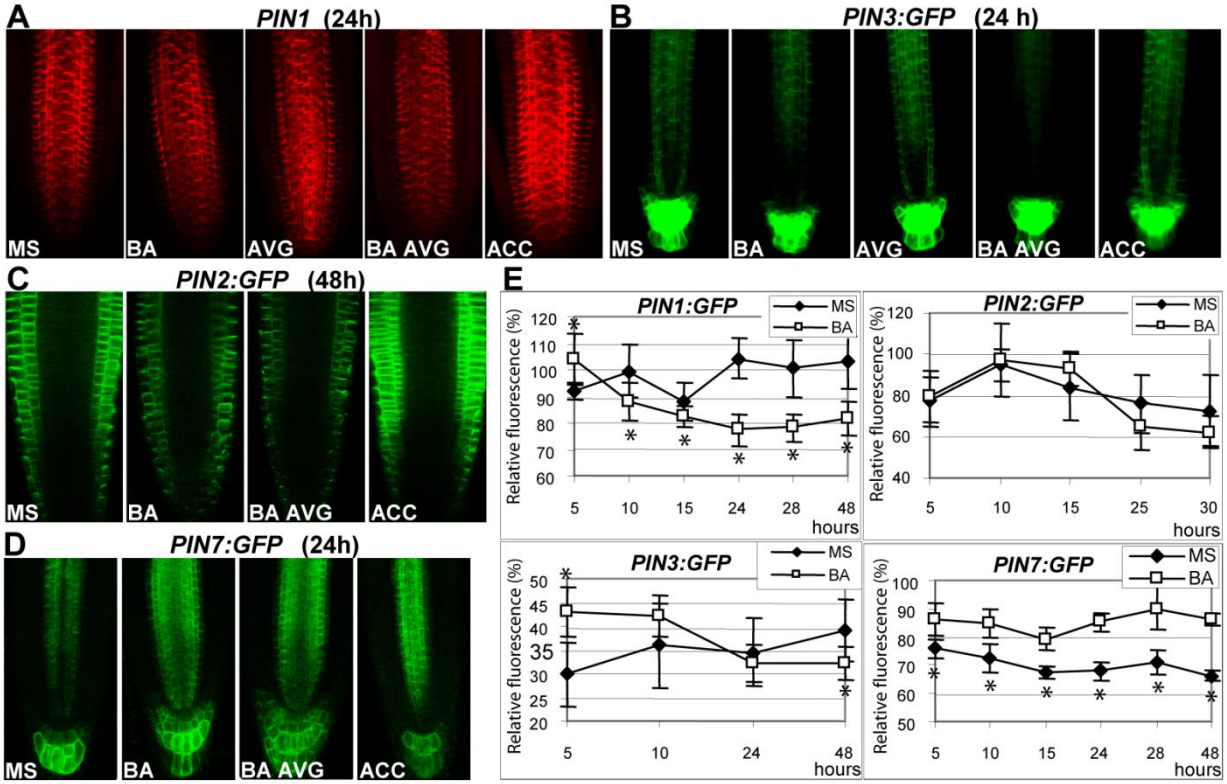

Fig. S3. CK effect on *PINs* expression. (A) Reduction of PIN1 signal (detected by PIN1-specific antibodies) by CK and stimulation by ACC. (B) Reduced *PIN3::GFP* expression after 24-h CK treatment. No significant effect of the ethylene precursor ACC was observed. (C) Reduction of *PIN2::GFP* after long-term (48 h) CK treatment in contrast to stimulatory effect of ACC. (D) CK-stimulated *PIN7:GFP* expression not affected by ACC. Six-day-old seedlings were incubated in control medium or supplemented with 5µM BA, 0.2 µM AVG, and 1 µM ACC. (E) Time-lapse expression analysis of PIN genes. Short time (4–5 h) CK treatment stimulates
PIN1 expression. From 10 h on, a strong inhibitory effect observed. Reduction of *PIN2:GFP* expression observed after 24-h treatment. *PIN3:GFP* expression stimulated by short-term (up to 10 h) CK treatment and down-regulated from 24 h on upon CK treatment. *PIN7:GFP* expression continuously stimulated by CK treatment. (*significantly different GFP fluorescence between MS-and CK-treated seedlings. Student's t test, $P<0.05$). Six-day-old seedlings incubated in control or with 5µM BA of supplemented media. MS (Murashige and Skoog) medium only; CK represented by BA; error bars represent SD.

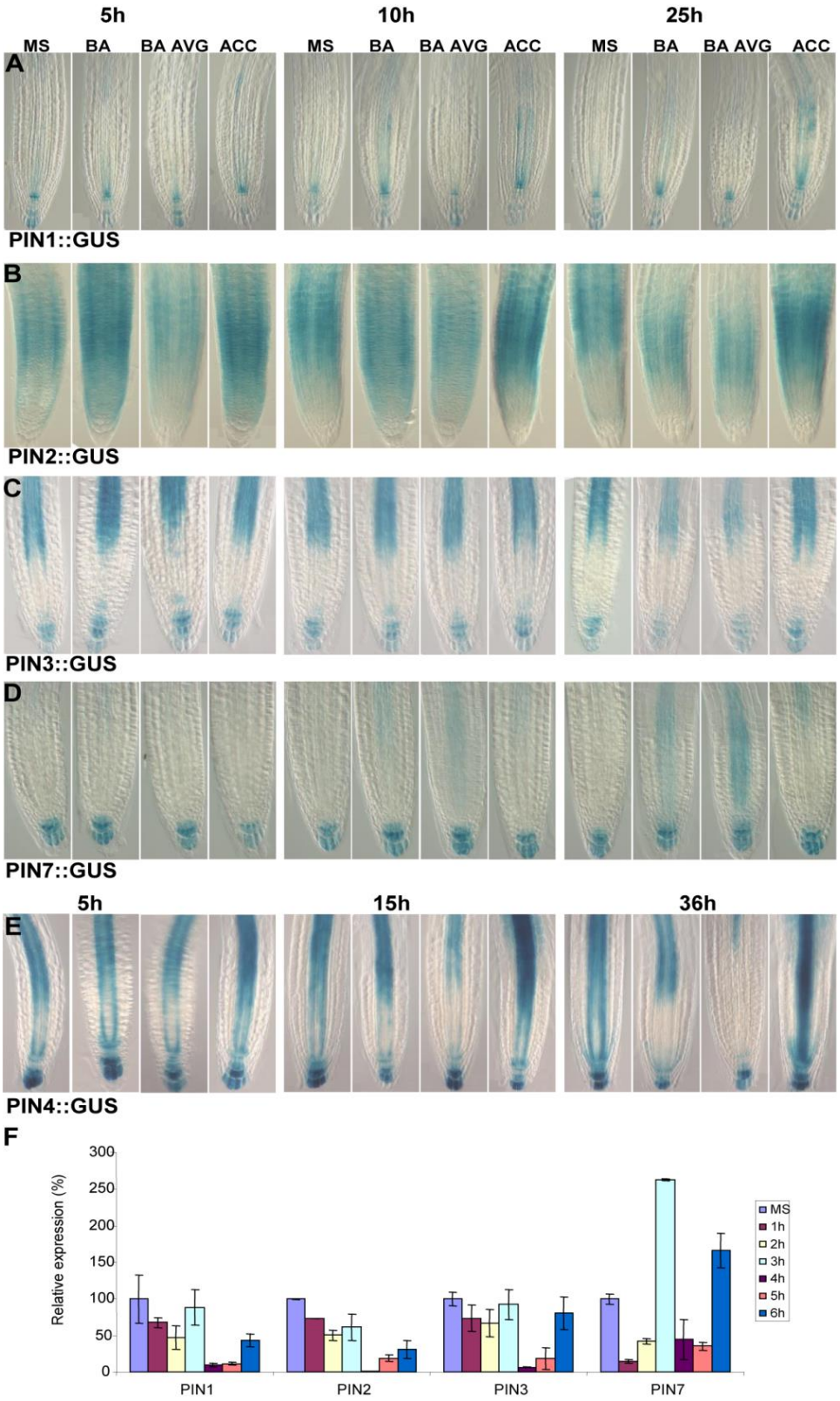

Fig. S4. Regulation of PIN genes transcription by CK. (A) CK interference with PIN1::GUS expression in an ethylene-dependent manner. From5hon PIN1::GUS expression is stimulated by CK and ACC. CK stimulatory effect is hampered when ethylene-biosynthesis inhibited. (B) Strong up-regulation ofPIN2::GUS expression by ACC. Moderate inhibition of PIN2 expression by CK after 25 h of treatment is observed at simultaneously diminished ethylene biosynthesis. (C) Induction and decrease of the PIN3::GUS expression after short (5 h) and long-term (25 h) CK treatment, respectively. No interference of ACC with PIN3 expression observed. (D)

Induction of the PIN7::GUS expression by CK from5hon without significant effect of ACC. (E) Down-regulation of PIN4::GUS expression by CK from 15 h on. ACC strongly interferes with CK effects on the PIN4 expression. Six-day-old seedlings incubated in medium supplemented with 5µM BA, 0.2 µM AVG, and 1µM ACC. (F) Time-lapse quantitative RT-PCR expression analysis showing differential regulation of PIN genes expression after CK treatment (10 M BA). MS (Murashige and Skoog) medium only; CK represented by BA.

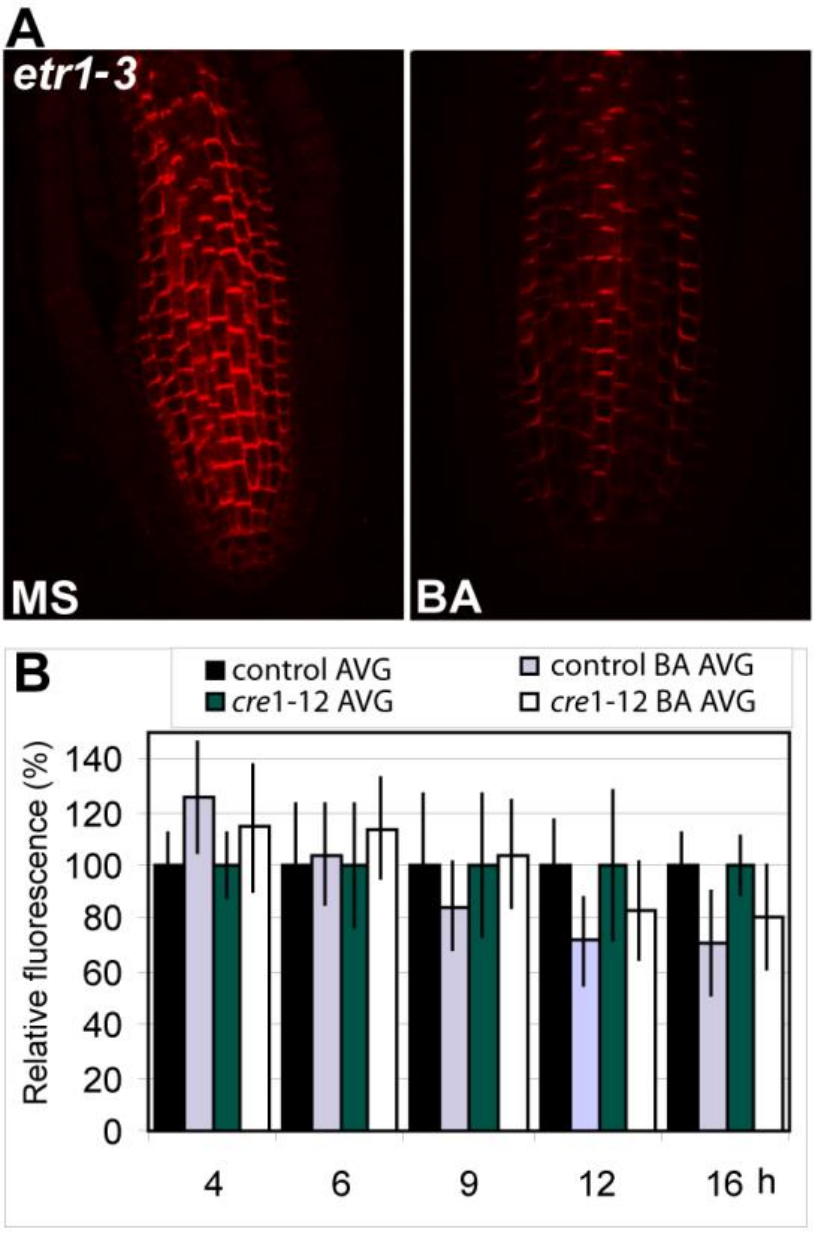

Fig. S5. Dependence of CK-mediated inhibition of PIN1 expression on CK but not on ethylene perception. (A) Reduced expression of *PIN1* in the CK-treated *etr1*–3 roots. (B) *PIN1::GFP* expression in *cre1–12* mutant less affected by CK treatment (significantly different from control treated 12 h with CK, Student's *t* test, $P<0.05$). Red signal, immunolocalization of PIN1 using PIN1-specific antibodies. Six-day-old seedlings incubated in control medium or supplemented with CK (5 µM BA, 0.2µM AVG) for 24 h (if not marked differently). RM, root meristem; MS, Murashige and Skoog medium only; CK represented by BA; error bars represent SD.

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Chapter 3.

The Cytokinin Response Factors regulate PIN gene transcription

Adapted from

The cytokinin response factors CRFs control PIN-like gene transcription
M. Šimášková J.A. Obrien, G. Van Noorden, A. Vieten, I. De Clercq, A. Van Haperen, C. Cuesta, K. Ötvös*, K. Hoyerova, S. Vanneste, P. Marhavy , F. Van Breusegem, A. Murphy, J. Friml, D. Weijers, T. Beeckman, and E. Benková (manuscript in preparation).*

M.S, and E.B initiated the project and designed most of the experiments, M.S carried out most of the experiments. J.A.O contributed to the generation of the trangenic mutant lines and phenotype analysis; A.V, G.V.N, I.D.C, S.V and A.V.H provided unpublished material; C.C assisted in protoplast assay, K.O contributed to the ChIP analysis, K.H performed auxin measurements and P.M set up the confocal time-lipse experimets. E.B, M.S and J.A.O analyzed and discuss the data. E.B and M.S wrote the manuscript. All authors saw and commented on the manuscript.

The Cytokinin Response Factors regulate *PIN* gene transcription

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Abstract

The plant hormones auxin and cytokinin are key endogenous plant growth regulators of which the activities are specified through a tightly coordinated crosstalk. Cytokinin modulation of the auxin distribution belongs to developmentally crucial interactions, but its molecular bases are largely unknown. We show that the cytokinin-mediated transcriptional control of *PIN-FORMED (PIN*) auxin efflux carriers occurs at specific *PIN CYTOKININ RESPONSE ELEMENT* (*PCRE*) promoter domains that, after removal, effectively uncouple the *PIN* transcription from the cytokinin regulation. The *PCRE,* but no other promoter region, is directly targeted by CYTOKININ RESPONSE FACTORS (CRFs), which are transcription factors of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family. Plants with modified CRF activity exhibit alterations in the *PIN* gene expression and developmental defects associated with impaired auxin distribution. We propose that CRF transcription factors are components of the transcriptional regulatory complex that acts in a specific domain of the *PIN* promoters to integrate cytokinin signals to fine-tune the *PIN* expression during plant growth and development.

Introduction

Auxin and cytokinin are among the key hormones that control plant growth and development. Both hormones contribute to the regulation of early embryogenesis[1–3] and various postembryonic organogenic processes, including lateral root organogenesis[4–7], shoot branching[8], phyllotaxis[9,10] , root apical meristem[11–14] , vasculature[15–17], and shoot apical meristem activity[10,18]. Recently, the principal pathways have been recognized that manage metabolism, distribution, and perception of both auxin and cytokinin and the backbone molecular components have been identified[19–22]. However, it became obvious that the auxin and cytokinin activities converged at many levels and that their crosstalk played an important role in fine-tuning the developmental output of the individual pathways. Well-established are the auxin-cytokinin mutual regulations of metabolic[23,24] and signaling pathways[3,10] as well as the cytokinin modulation of the auxin transport[7,14,25–28]. Cytokinin at both transcriptional and posttranslational levels affects the expression of PIN-FORMED (PIN) auxin efflux carriers, one of the key components of the polar auxin transport machinery[14,26,27,29]. Through the cytokinin receptor ARABIDOPSIS HISTIDINE KINASE3 (AHK3) and the downstream signaling components ARABIDOPSIS RESPONSE REGULATOR (ARR1) and ARR12, cytokinin has been proposed to directly stimulate the expression of AUXIN-INDUCED/SHORT HYPOCOTYL (IAA3/SHY2) that acts as an auxin signaling repressor. Consequently, due this repression, IAA3/SHY2 might attenuate the auxin-dependent transcription of *PIN* genes[26]. However, thus far, the components of the transcriptional complex that directly controls *PIN* transcription in response to cytokinin are unknown.

Here, we identified specific promoter domains required for the cytokinin-dependent transcriptional control of *PIN1* and *PIN7* and show that their deletions effectively uncouple the *PIN* transcription from the cytokinin regulation. Expression of *PIN*s in a cytokinin-independent manner affects plant development and its cytokinin sensitivity. We found that the 200-bp nucleotide domains, designated as *PIN CYTOKININ RESPONSE ELEMENT1* (*PCRE1*) and *PCRE7*, but no other promoter regions, are directly targeted by the CYTOKININ RESPONSE FACTORs (CRFs), which are transcription factors of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family[30]. Plants with modified CRF activity exhibit alterations in the *PIN* expression and developmental defects associated with impaired auxin distribution. We propose that the CRF transcription factors are components of the transcriptional regulatory complex that acts on the specific domain of *PIN*

promoters to integrate cytokinin signals to fine-tune the *PIN* expression during plant growth and development.

Results and Discussion

Deletion of the 200-bp promoter domain results in cytokinin-insensitive expression of PIN *genes*

The cytokinin-controlled expression of *PIN* genes is a developmentally important mechanism that regulates the cell-to-cell auxin transport and auxin distribution through the plant body[14,26]. As previously shown, increases in cytokinin levels attenuate the transcription of *PIN1*, *PIN3*, and *PIN4*, but promote that of *PIN7*[14,15]. To identify the control elements required for this cytokinin-dependent *PIN* transcription, a promoter deletion analysis was used. The promoter sequences of *PIN1* and *PIN7*, 2098 bp and 1423 bp upstream from the translational start site (ATG), respectively, were progressively truncated by 200 bp and fused to the green fluorescent protein (GFP) reporter gene. The truncated constructs, designated as *ΔPIN1::GFP1/10* to *1/1* and *ΔPIN7::GFP7/7* to *7/1*, were introduced into *Arabidopsis thaliana* plants (Chapter 3 [Figure 1A, B) and their cytokinin sensitivity was examined. In agreement with previous findings, cytokinin attenuated and up-regulated the expression of *ΔPIN1::GFP1/10* and *ΔPIN7::GFP1/7,* respectively, thereby confirming that these promoter regions are sufficient to mediate the hormonal response (Figure 1A, E). Subsequent removal of 200-bp nucleotides from the *PIN1* promoter did not affect the cytokinin sensitivity of the *ΔPIN1::GFP1/9, ΔPIN1::GFP1/8,* and *ΔPIN1::GFP1/7* constructs. Yet, from *ΔPIN1::GFP1/6* on, all consecutive truncations caused dramatic decreases in the cytokinin sensitivity (Figure 1A, B; Chapter 3[Figure 2]). The abrupt change in the cytokinin response as a consequence of a 200-bp element deletion between *ΔPIN1::GFP1/7* to *ΔPIN1::GFP1/6* indicated that within 1417 bp and 1212 bp upstream from the ATG region, *PIN1 CYTOKININ RESPONSE ELEMENT1* (*PCRE1*) might be present that is required for cytokinin-mediated transcriptional control. Similarly, the loss of 200 bp between *ΔPIN7::GFP7/7* and *ΔPIN7::GFP7/6* compromised the cytokinin sensitivity of the *PIN7* promoter; the corresponding sequence between 1423 bp and 1223 bp upstream of ATG was designated *PCRE7* (Figure 1E, F; Chapter 3 [Figure 3]). (Note: all constructs missing the *PCRE* element are hereafter indicated as *ΔPIN*).

The role of *PCRE1* and *PCRE7* in the cytokinin-sensitive expression was further tested with the *PIN1-GFP* and *PIN7-GFP* translational constructs driven by the truncated *ΔPIN1* and *ΔPIN7* promoters, respectively. Quantification of the membrane *PIN-GFP* signal demonstrated that the expression of both *PIN1-GFP* and *PIN7-GFP*, when driven by truncated promoters, was largely insensitive to the cytokinin treatment in the cells of the central root cylinder (Figure 1C, D, I and G, H, J, respectively) and in the initials of lateral root primordia (Supplementary Figure 1A-D, E and F-J, respectively).

Close examination of the expression in *ΔPIN1::GFP, ΔPIN1::PIN1-GFP, ΔPIN7::GFP*, and *ΔPIN7::PIN7-GFP* lines revealed that, besides changes in the cytokinin sensitivity, the lack of either *PCRE1* or *PCRE7* affected the tissue-specific pattern of the respective *PIN* gene expression. The activity of the *ΔPIN1* promoter was significantly higher in the root provasculature, but lower in endodermal cells than that of the full *PIN1* promoter (Figure 1A, C compared to B, D, insets, and I). By contrast, the *ΔPIN7* promoter activity was strongly weakened in the root provasculature and columella cells when compared to the full *PIN7* promoter (Figure 1E, G compared to F, H, and J).

In summary, the loss of cytokinin sensitivity as the consequence of *PIN1* and *PIN7* promoter truncations implies the presence of the specific regulatory element on which the cytokinin-susceptible transcriptional complex might act to fine-tune the *PIN* expression in response to cytokinin. Noteworthy, altered expression patterns of the *ΔPIN1* and *ΔPIN7* promoters indicate that cytokinin might, through these regulatory elements, participate in the establishment and maintenance of the proper *PIN* expression pattern.

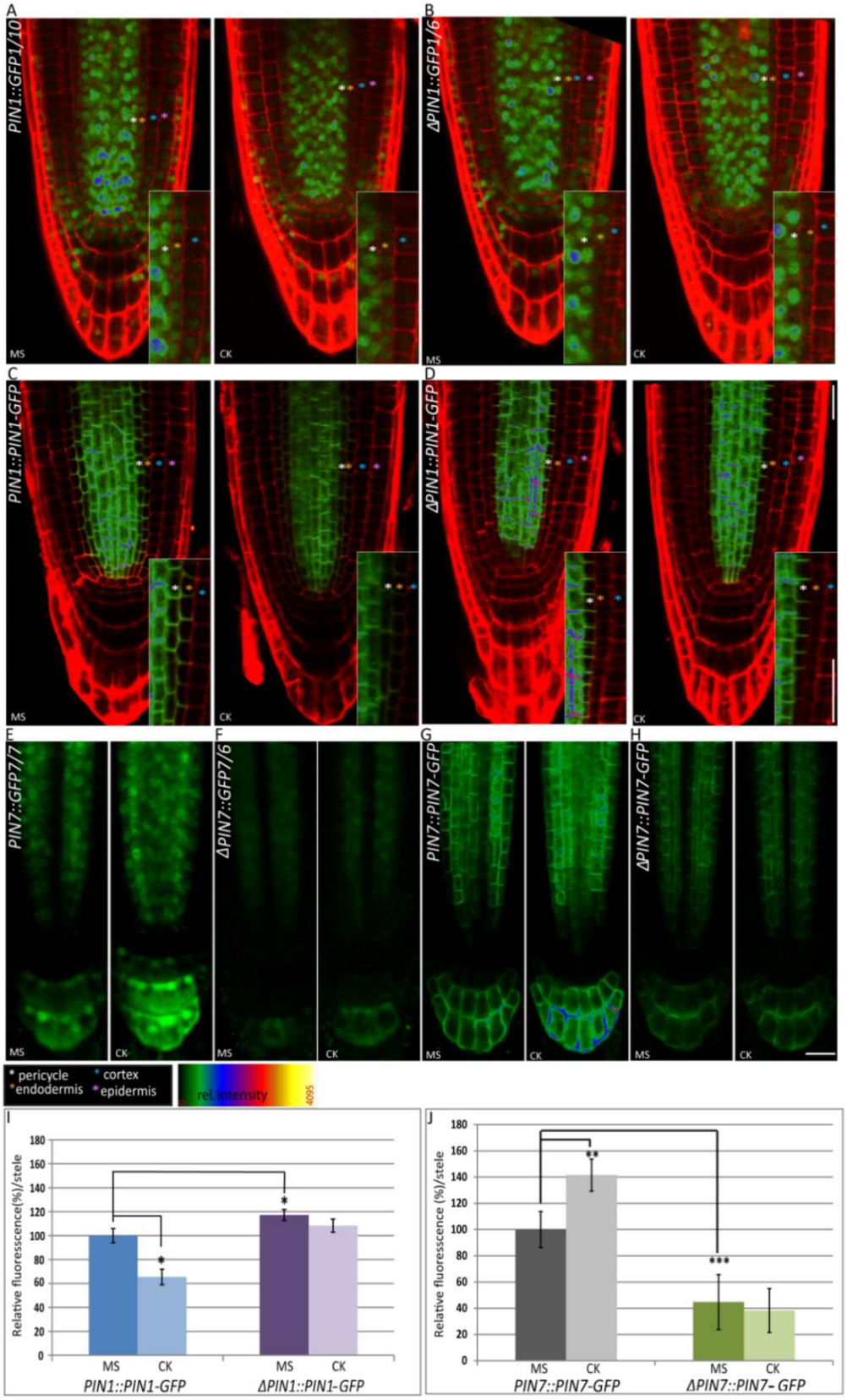

Figure 1. ***Truncation of PIN promoter results in cytokinin-insensitive PIN transcription.*** (A-D) Expression of GFP reporter (A, B) and PIN1-GFP (C, D) translation fusion is down-regulated after cytokinin treatment when driven by the full *PIN1* (A,C), but not when driven by the truncated *ΔPIN1* promoter (B, D). (E-H) Expression of GFP reporter (E, F) and PIN7-GFP (G, H) translation fusion is up-regulated in response to cytokinin when

driven by full the *PIN7* (E,G), but not when driven by the truncated *ΔPIN7* promoter (F,H). Color-coded asterisks indicate the different root cell files. A semi-quantitative color-coded heat-map of the GFP fluorescence intensity is provided. Green, nuclear-localized GFP reporter (A-D), membrane localized PIN1-GFP (C, D), and PIN7-GFP (E-H); red, propidium iodide staining. Scale bar 20µm. (I, J) Quantification of PIN1-GFP (I) and PIN7-GFP (J) expression when driven by full or truncated promoters in the provasculature of primary roots, respectively. Roots of 7-day-old seedlings (n=15) were treated with control Murashige Skoog (MS) medium without or with cytokinin (CK) 5 µM N^6-benzyladenine for 8 h. Student's *t* test (*p<0.05, **p<0.01; p<0.001 ***, n= 15). Error bars represent standard error.

Uncoupling PIN1 and PIN7 transcription from the cytokinin control modulates plant development and sensitivity to cytokinin

Part of the cytokinin effect on plant growth and development has been proposed to be mediated through the cytokinin-controlled *PIN* expression[7,14,26]. Hence, the identification of the cytokinin regulatory elements in *PIN* promoters provides a powerful tool to examine the significance of the cytokinin-dependent *PIN* transcription in plant developmental processes.

To dissect the developmental role of cytokinin-regulated *PIN* expression, plants expressing *PIN1-GFP* and *PIN7-GFP* under the control of truncated cytokinin-insensitive promoters were thoroughly inspected. Real-time analyses revealed that in young up to 10-day-old seedlings growth of roots expressing either *PIN1::PIN1-GFP* or *ΔPIN1::PIN1-GFP* was mutually indistinguishable, but from day 11 on, that of the *ΔPIN1::PIN1-GFP* roots progressed significantly faster than that of control roots (Figure 2A, E). Furthermore, uncoupling the *PIN1-GFP* expression from the cytokinin control promoted lateral root initiation (Figure 2A, F). In all inspected parameters, such as root growth, root meristem size, and lateral root initiation, the *ΔPIN1::PIN1-GFP* plants exhibited a significantly reduced cytokinin sensitivity (compare Figure 2A to B; and E, F, and G).

Similarly to *ΔPIN1::PIN1-GFP*, expression of *ΔPIN7::PIN7-GFP* enhanced root growth when compared to control plants (Figure 2C, H). Attenuation of the *PIN7* cytokinin-mediated transcription interfered with the cytokinin sensitivity of root growth (Figure 2D, H), root meristem (Figure 2J), and lateral root initiation (Figure 2D,I).

Altogether, these data support the role of the *PCRE* elements in fine-tuning the *PIN* expression and show that cytokinin-controlled transcription of *PIN* genes through *PCRE* elements is important for proper plant growth and development.

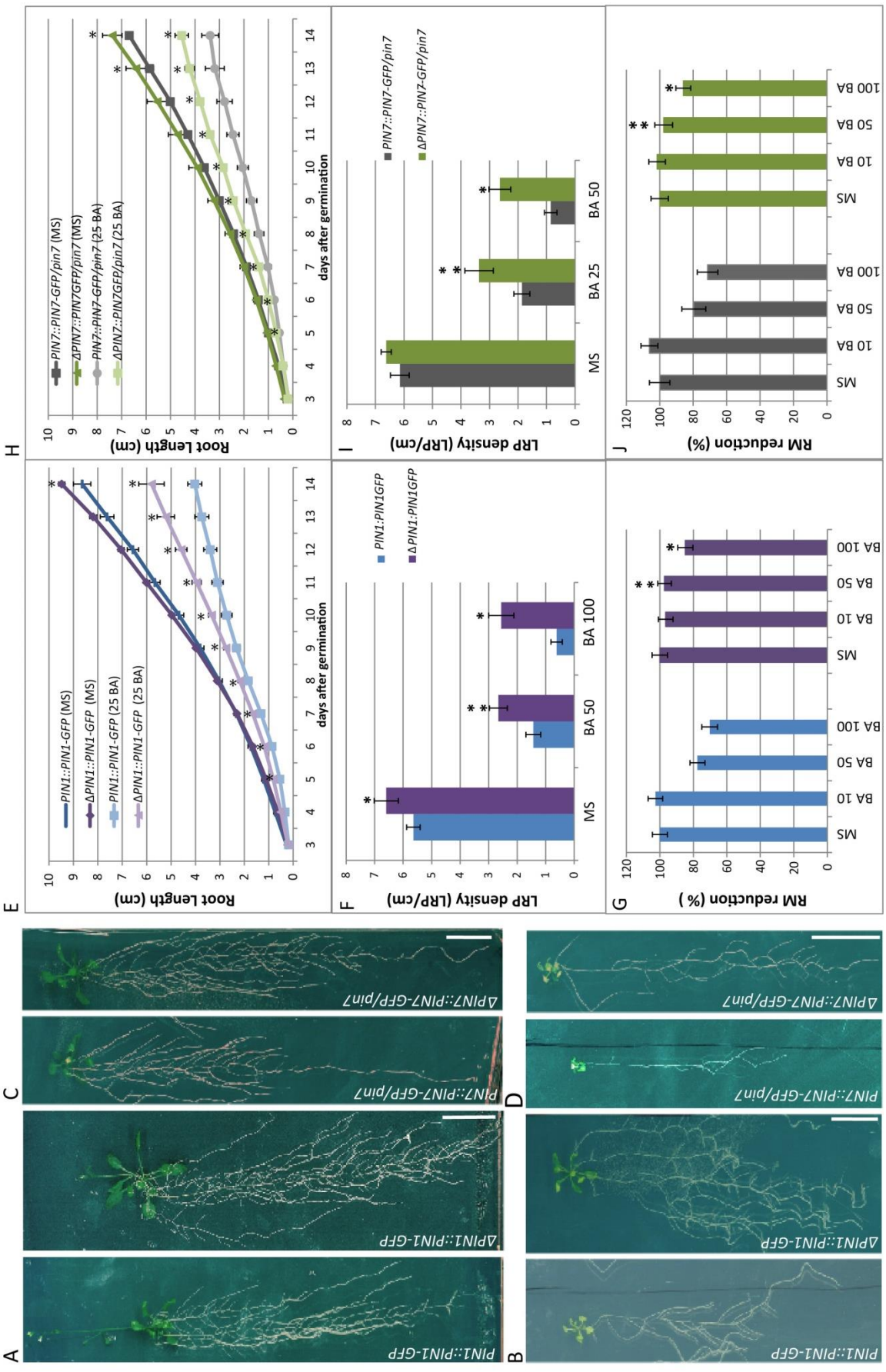
A
B
C
D
E
F
G
H
I
J
PIN1::PIN1-GFP
ΔPIN1::PIN1-GFP
PIN7::PIN7-GFP/pin7
ΔPIN7::PIN7-GFP/pin7
Root Length (cm)
days after germination
PIN1::PIN1-GFP (MS)
ΔPIN1::PIN1-GFP (MS)
PIN1::PIN1-GFP (25 BA)
ΔPIN1::PIN1-GFP (25 BA)
PIN7::PIN7-GFP/pin7 (MS)
ΔPIN7::PIN7GFP/pin7 (MS)
PIN7::PIN7-GFP/pin7 (25 BA)
ΔPIN7::PIN7GFP/pin7 (25 BA)
LRP density (LRP/cm)
PIN1:PIN1GFP
ΔPIN1::PIN1GFP
MS
BA 50
BA 100
PIN7::PIN7-GFP/pin7
ΔPIN7::PIN7-GFP/pin7
BA 25
RM reduction (%)
BA 10
BA 50
BA 100

Figure 2. ***Uncoupling of PIN transcription from the cytokinin control affects plant development***. (A, B, E-G) *PIN1-GFP* expression under cytokinin-insensitive *ΔPIN1* truncated promoter results in enhanced root growth (A, E) and reduced cytokinin sensitivity of primary roots (E), lateral root initiation (F), and root meristem size (G) when compared to control *PIN1::PIN-GFP* seedlings. (C, D, H-J) *PIN7-GFP* expression under cytokinin-insensitive *ΔPIN7* promoter results in enhanced root growth (C, H) and reduced cytokinin sensitivity of root growth (D, H), lateral root initiation (I), and root meristem size (J) when compared to control *PIN7::PIN7-GFP* seedlings. Seedlings were grown for 28 days (A-D), 14 days (E, H), 10days (F), and 7days (G, I, J) on control Murashige Skoog (MS) medium without or with cytokinin (CK) 0.25 μM N^6-benzyladenine, if not specified differently. Student's *t* test (*p<0.05; **p<0.01 root growth (n=12), root meristem, and lateral root density measurements (n=15). Error bars represent standard error. Scale bar, 2 cm. Similar results were obtained when a second independent transgenic line was used for the experiments.

Cytokinin response factors (CRFs) control PIN transcription by direct interaction with the PCRE elements

To identify the upstream regulatory factors that regulate *PIN1* and *PIN7* transcription by direct interaction with *PCRE1* and *PCRE7*, respectively, by means of a yeast 1-hybrid (Y1H) assay, we screened the REGIA open reading frame (ORF) library that contains a set of ~1300 *Arabidopsis* transcription factors (TFs) and transcriptional regulators[31]. In the Y1H screen with *PCRE1* and *PCRE7* as bait, CRF2, CRF3, and CRF6 were identified that belong to the cytokinin-inducible subset of the AP2/ERF TF family (Figure 3A, B). To confirm that the CRFs physically interact with *PCRE1* and *PCRE7* (*in planta*), we analyzed the *CRF2::CRF2-GFP* and *35S::CRF6-GFP* transgenic plants with a chromatin immunoprecipitation (ChIP) followed by quantitative PCR assays (ChIP-qPCR). Chromatin immunoprecipitated with anti-GFP antibodies was profoundly enriched in the *PCRE1* and *PCRE7* regions of the *PIN1* and *PIN7* promoters, respectively. No enrichment was detected when other randomly selected sequences in the *PIN1* (*PIN1* [-512 -433]) and *PIN7* (*PIN7* [*-553-357*]) promoters were tested (Figure 3C, D). In other words, CRFs interact *in vitro* and *in planta* (CRF2 and CRF6) with the *PCRE1* and *PCRE7* elements in the *PIN1* and *PIN7* promoters, respectively.

To get insights into the role of the CRFs in the regulation of the *PIN* gene transcription, we performed a transient expression assay in *Arabidopsis* protoplasts. The expression of both *PIN1::LUCIFERASE* (*LUC*) and *PIN7::LUC* reporters was strongly activated when co-expressed with *CRF2* and *CRF6*, and but not with *CRF3* driven by the constitutive *35S* promoter (Figure 3E-G). Interestingly, the *PIN1::LUC* expression increased significantly when protoplasts isolated from plants grown at constant light were transfected (Supplementary Figure 2A). Thus, *CRF3* might be part of a complex, light-regulated transcriptional machinery. Importantly, the *LUC* reporter expression did not or slightly increase when driven by the truncated *ΔPIN1* and *ΔPIN7* promoters, further confirming the

importance of *PCRE1* and *PCRE7* for the CRF-dependent transcription (Figure 3F-H). Collectively, these data indicate that CRFs through direct interaction with specific domains within the *PIN1* and *PIN7* promoters contribute to the transcriptional control of these genes.

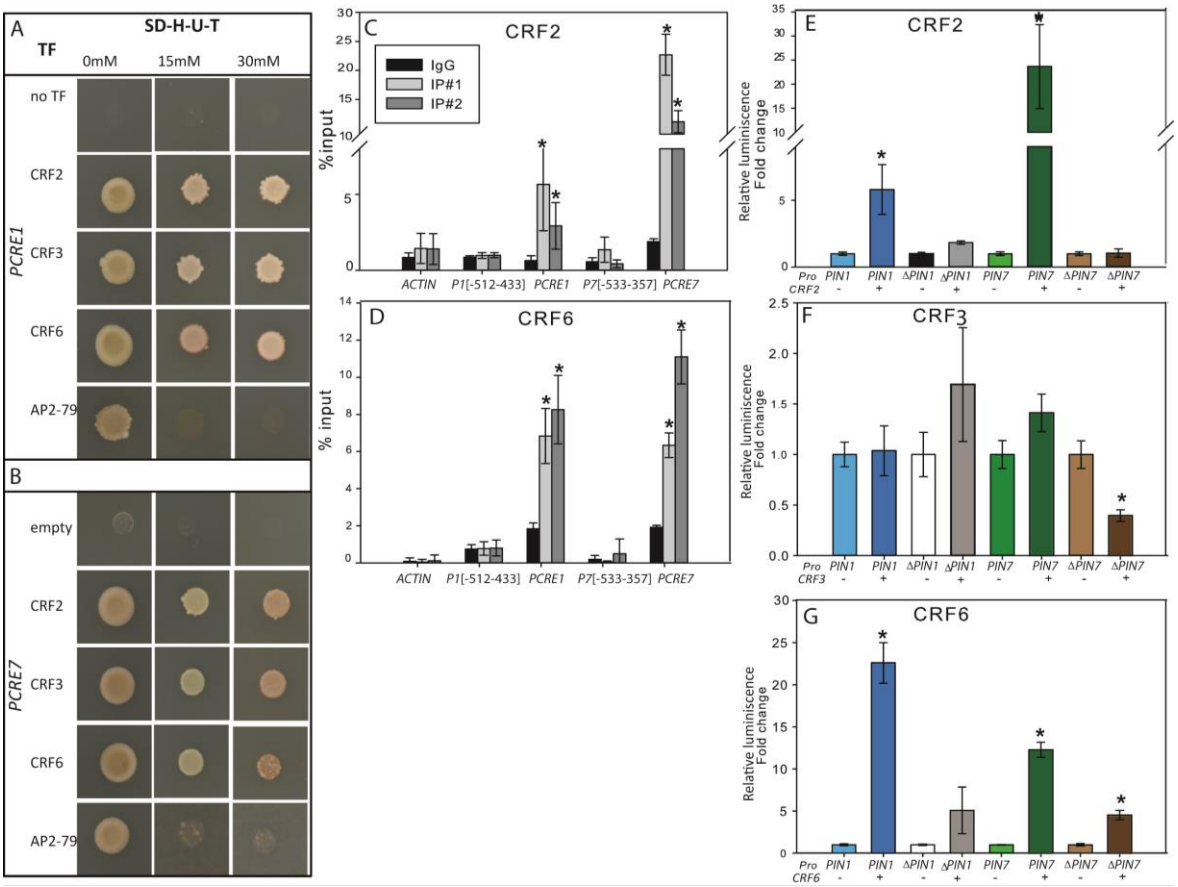

Figure 3. ***Cytokinin response factors (CRFs) interact with PCRE elements.*** (A, B) CRF2, CRF3, and CRF6 interaction with *PCRE1* (A) and *PCRE7* (B) elements results in HIS3 reporter activation in Y1H assays in contrast to AP2-79 that does not interact. Yeasts were grown on SD-H-U-T minimal media without histidine (H), uracil (U), and tryptophan (T) supplemented with 3-amino-1,2,4-triazole (3AT). (C, D). ChIP showing interaction of CRF2 (C) and CRF6 (D) TFs with *PCRE1* and *PCRE7* and enrichment with anti-GFP antibody, but not with the *PIN1* (-512 -433) and *PIN7* (-553-357) elements. (E-G) Significantly up-regulated expression of LUCIFERASE reporter driven by either the *PIN1* or *PIN7* promoter by co-expression with *CRF2* (E) and *CRF6* (G), but not with *CRF3*(F) in *Arabidopsis* mesophyll protoplasts, nor driven by their truncated counterparts *(ΔPIN1*, *ΔPIN7*). Student's *t* test (*p<0.05, n=3). Error bars represent standard error (protoplast assay) and standard deviation (ChIP).

Expression of PIN1 and PIN7 genes is altered in crf mutants

The initial expression analyses revealed that the CRFs and the *PIN1* and *PIN7* expression patterns largely overlap in roots (compare Supplementary Figure 3 with Figure 1 A, E), supporting their role as direct transcriptional regulators. To evaluate the impact of the CRFs on the *PIN* expression *in planta*, we examined lines with an altered *CRF* expression. Analysis

with *PIN1::GFP* and *PIN1::PIN1-GFP* reporter constructs and immunodetection revealed a significant reduction in *PIN1* expression in *crf2* and *crf3* and *crf3crf6* multiple loss-of-function mutants, but an enhancement in the *crf6* background (Figure 4A-D). Vice versa, the *PIN1* expression was significantly up-regulated in roots overexpressing either *CRF2* or *CRF3,* but reduced in *35S::CRF6* roots (Figure 5A-D and Supplementary Figure 4A-C). Deletion of the *PCRE1* element in the *ΔPIN1::PIN1-GFP* line interfered with the stimulating effect of *CRF2* and *CRF3* on the *PIN1* expression, demonstrating the significance of this element for the expression mediated by *CRF2* and *CRF3* (Figure 5E, F). When the *PIN7* expression was examined with the *PIN7::GUS* and *PIN7::PIN7-GFP* reporter constructs, it was, as *PIN1,* significantly down-regulated in *crf3* and up-regulated in *crf6* loss-of-function mutants (Figure 4E-G). The deviations were more pronounced in *CRF* gain-of-function transgenic lines. Enhanced *PIN7* expression was detected in *RPS5A::CRF2* roots, but, unlike *PIN1*, *PIN7* was down-regulated and not up-regulated in *35S::CRF3* plants (Figure 5G, H and Supplementary Figure 4E, F). Overexpression of *CRF6* attenuated the expression of *PIN7* in columella, but enhanced it in the central cylinder (Figure 5G, H; Supplementary Figure 4G). Nevertheless, lack of *PCRE7,* as in the case of *PIN1,* abolished the *PIN7* up-regulation by *CRF2* (Figure 5J).

Altogether, these data show that CRFs *in planta* contribute to balancing the *PIN* expression and CRF homologs might have specific functions in the control of the *PIN1* and *PIN7* expression. Yet, *in planta,* the *PIN* expression patterns are not fully consistent with the protoplast assays that implicate CRFs as positive regulators of *PIN* transcription. In particular, the CRF6 activity in root differs from that suggested by protoplast assays, possibly due to a more complex situation *in planta*, where developmentally context-specific conditions might contribute to the output. Recently, interactions have been demonstrated of individual CRFs with other family members as well as with type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs)[32]. Thus, CRFs might act as part of a transcriptional complex in which additional components might function as modifiers. Such a scenario is supported by the results from the protoplast assay that revealed the light-dependent character of the CRF3-mediated regulation. Noteworthy, when co-expressed, *CRF3* significantly attenuated the positive effect of *CRF6* on the *PIN1::LUC* expression (Supplementary Figure 2B), further confirming the multicomponent regulation of *PIN* transcription.

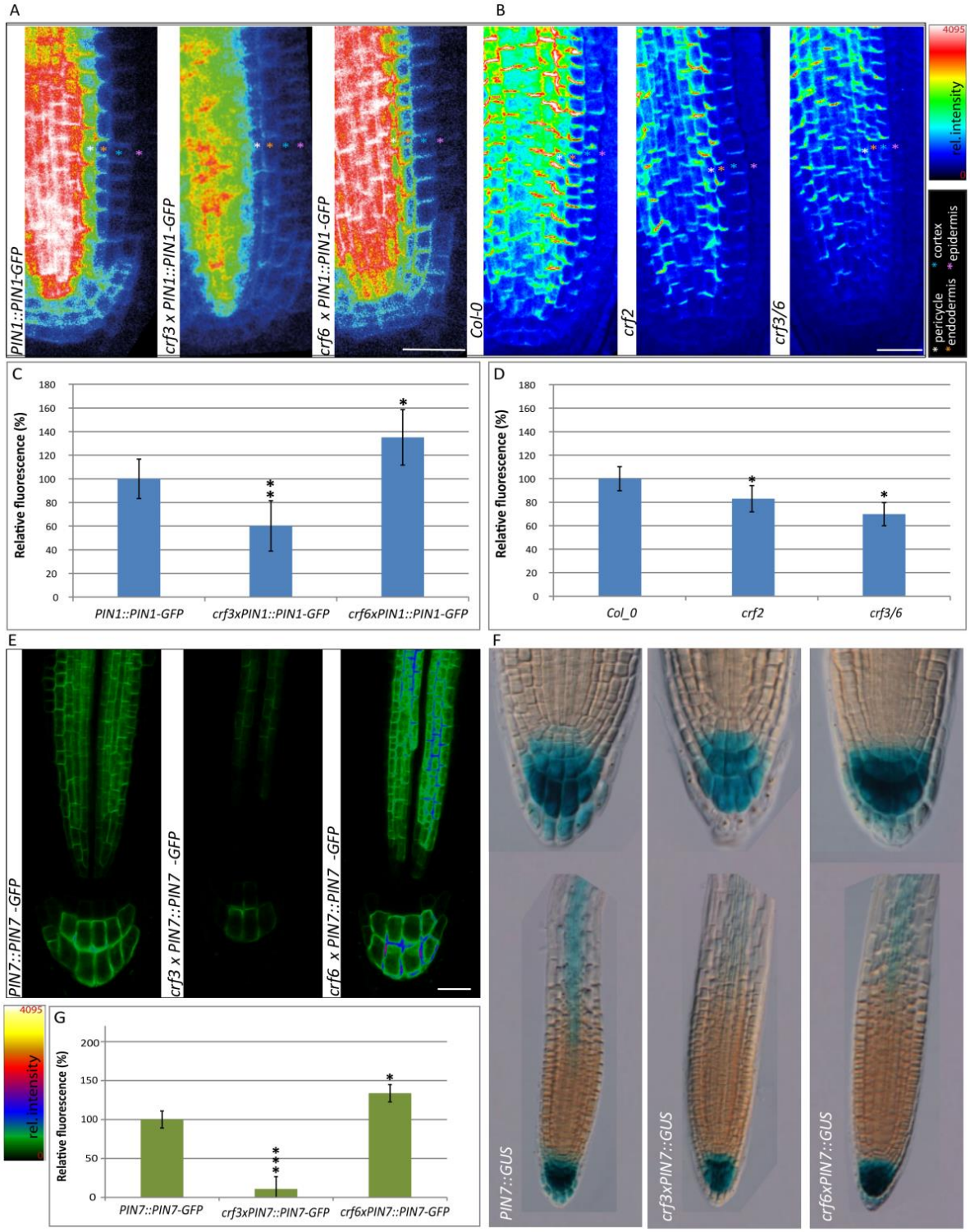

Figure 4. ***Expression of PIN1 and PIN7 is altered in crf loss-of-function mutants.*** (A-D) PIN1 expression is down-regulated in *crf2* and *crf3* and multiple *crf3crf6* loss-of-function mutants, but up-regulated in *crf6* mutants. *PIN1* expression was monitored with *PIN1::PIN1-GFP* (A) and immunolocalized with a PIN1-specific antibody (B). Quantification of the PIN1 membrane signal in endodermis cells (C, D). (E-G) *PIN7* expression is down-regulated in *crf3* and up-regulated in *crf6* loss-of-function mutants. *PIN7* expression was monitored with *PIN7::PIN7-GFP* (E) and *PIN7::GUS* (F) reporter constructs. Quantificiation of the PIN7-GFP membrane signal in columella cells (G). Student's *t* test (*p<0.05, **p<0.01; p<0.001 ***, n=10-15). Error bars represent standard error. Analysis of 7-day-old (A-G) and 5-day-old (F) seedlings. Color-coded asterisks indicate the different root cell files. A semi-quantitative color-coded heat-map of the GFP fluorescence intensity is provided.

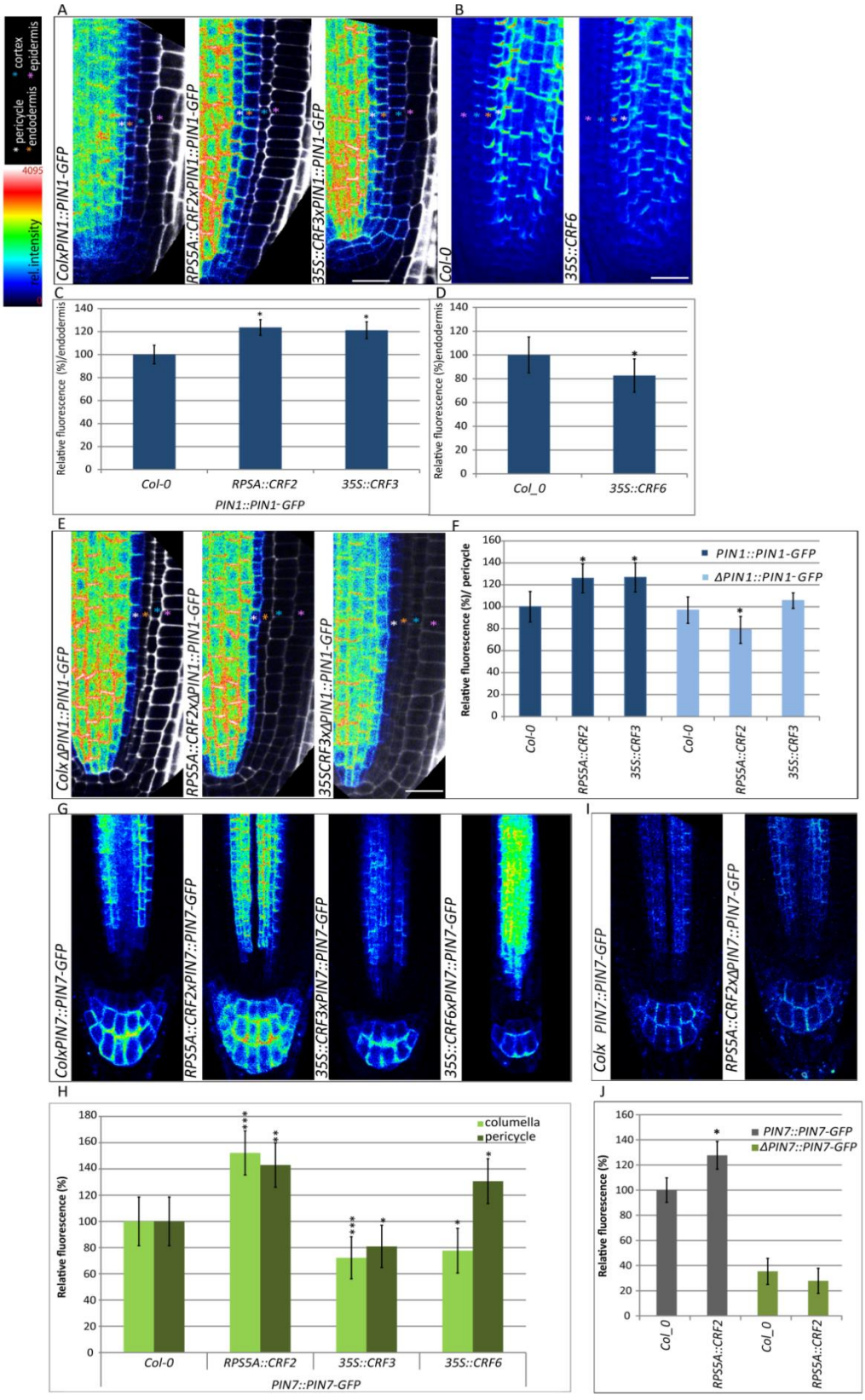

Figure 5. ***Expression of PIN genes is altered in CRF-overexpressing lines.*** (A-D) *PIN1* expression is up-regulated in *CRF2* and *CRF3*, but down-regulated in *CRF6*-overexpressing lines. *PIN1* expression was monitored with *PIN1::PIN1-GFP* (A) and immunolocalized with PIN1-specific antibody. (B) Quantification of PIN1 membrane signal in the endodermis. Student's *t* test (*p<0.05, n=15) (C, D). Color-coded asterisks indicate the different root cell files. A semi-quantitative color-coded heat-map of the GFP fluorescence intensity is

provided. (E, F) *PIN1-GFP* driven by truncated *ΔPIN1* promoter is not up-regulated in *CRF2* and *CRF3*-overexpressing line. Quantification of the PIN1 membrane signal in pericycle cells (F). Student's *t* test (*p<0.05, n =10-15). (G, H) Up-regulation of *PIN7* expression in pericycle cells of lines overexpressing *CRF2* and *CRF6*, but down-regulation in *CRF3*-overexpressing line, in contrast to columella cells, where *PIN7* expression is attenuated in lines overexpressing *CRF3* and *CRF6*. (I, J) No up-regulation of *PIN7-GFP* driven by the truncated *ΔPIN7* in the *CRF2*-overexpressing line. Quantification of PIN7-GFP membrane signal in pericycle (H,J) and columella cells (H). Student's *t* test (*p<0.05, **p<0.01; p<0.001 ***, n =15-20). Error bars represent standard error. Analysis was performed on 7-day-old seedlings.

Loss-of-function mutants of CRFs exhibit an auxin transport-defective phenotype

Altered expression of *PIN1* and *PIN7* in *crf* loss-of-function mutants might result in an abnormal auxin distribution and, consequently, in developmental and patterning defects as previously demonstrated for auxin distribution mutants[2,11]. Indeed, auxin measurements in root tips of *crf3crf6* mutants revealed a correlation between the reduced expression of *PIN1* and *PIN7* and an increase in auxin levels (Figure 6A).

By closer examination of plants lacking the CRF activity, developmental abnormalities were found reminiscent of those caused by impaired auxin transport. The number of defects was significantly enhanced, such as abnormal division of upper suspensor cells, embryo proper, and, occasionally, double embryos, in embryos of *crf2*, *crf3*, and *crf3crf6* loss-of-function mutants when compared with control lines (Figure 6B-H), thus, phenocopying the *pin1*, *pin7*, and multiple *pin* embryo defects[2]. Accordingly, *CRF2*, *CRF3*, and *CRF6* expression was detected in early embryos (Supplementary Figure 3). At the seedling level, the embryo patterning defects resulted in an increased proportion of seedlings with an abnormal cotyledon number (1 or 3) or phyllotactic problems, when just one leaf would be initiated (Figure 6I-L). Furthermore, absence of the *crf2*, *crf3*, and *crf3crf6* mutants correlated with dramatic reductions in root length, root meristem size, and lateral root initiation (Figure 6M-R), but *crf6* loss-of-function mutants with an enhanced *PIN1* and *PIN7* expression exhibited enlarged roots and root meristem (Figure 6M-R).

Altogether, lack of the CRF activities alters auxin accumulation in the root tips and leads to many developmental defects, mimicking phenotypes of auxin distribution mutants[2,11,33]. These observations strongly support the role of CRFs as regulators of auxin transport through the direct control of the *PIN* expression.

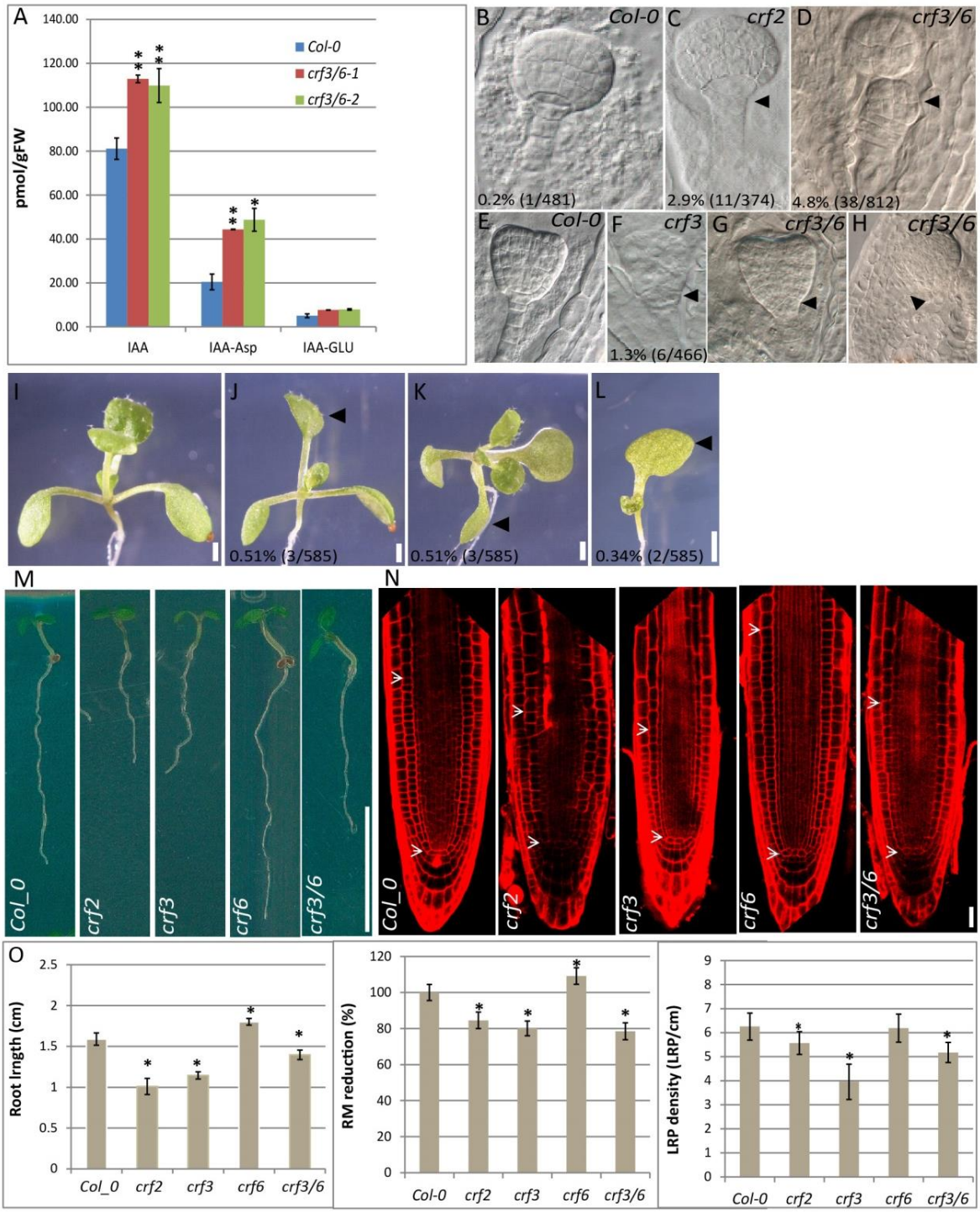

Figure 6. ***Crf mutants exhibit defective embryogenesis and root development.*** (A) Auxin accumulation in the root tip of *crf3crf6* multiple mutants. (B-H) Abnormal division of upper suspensor cells, embryo proper, and, occasionally, double embryos in embryos of *crf2* (C), *crf3* (F), and *crf3crf6* (D, G, H) loss-of-function mutants when compared with the control (B, E). (I-L) Increased proportion of abnormal cotyledons (K, L) or phyllotactic problems (J) in *crf3crf6* seedlings. Arrowheads indicate developmental defects. (M-R) Lack of *crf2*, *crf3*, *crf6* and *crf3crf6* function correlated with changes in root length (M, O), root meristem size (N, P), and lateral root primordia density (R). Seedlings were grown for 7 days on control Murashige and Skoog medium (M-R). Student's *t* test (*p<0.05; n=10-15). Error bars represent standard error. Scale bars (I-L) 1 mm, (N) 2 cm, (O) 20 µm.

Conclusions

Auxin distribution and formation of auxin gradients are among the conserved universal mechanisms to control plant organogenesis. Modulation of the transport machinery activity that regulates the auxin movement in plants directly impacts on plant development and, thus, represents a developmentally efficient tool to flexibly adapt plant architecture according to the changing environmental conditions. Exogenous factors, such as light or gravity, as well as endogenous factors, such as the plant hormones ethylene, gibberellin, jasmonate, and cytokinin execute part of their regulatory role through the interaction with the polar auxin transport machinery[14,26,29,34–39]. Ethylene, jasmonate, and cytokinin have been demonstrated to modulate the auxin transport through the transcriptional control of the PIN auxin efflux carriers[26,35,38,40,41], but, until now, the insights into the molecular mechanisms underlying this regulation are dispersed. Detailed promoter analyses of *PIN1* and *PIN7* genes that had been shown previously to be under cytokinin transcriptional control led to identification of *PCRE* elements, of which the deletion resulted in cytokinin-insensitive *PIN* gene transcription. Uncoupling of the *PIN* expression from the cytokinin control affects primary and lateral root growth, implying that a tight cytokinin-controlled *PIN* expression is important for normal plant growth and development. Interestingly, changes in root growth rates, particularly pronounced in older seedlings, indicate that cytokinin-dependent *PIN* transcription might play a role in the adjustment of root growth during plant aging. By Y1H screening for *PCRE*-interacting TFs, CRF2, CRF3, and CRF6 TFs were detected and interaction of CRF2 and CRF6 with *PCRE* was confirmed *in planta* by ChIP assays. Currently, an *in planta* interaction (ChIP) for CRF3 could not be studied yet, due to the lack of a proper *CRF3-GFP* transgenic line. Originally, CRFs were identified as a subgroup of AP2 TFs, of which the transcription is induced by cytokinin, and had been proposed to mediate the transcriptional response to cytokinin together with type-B ARRs[42]. In this light, the characterized *PIN* genes as direct targets of CRFs represent the missing link between the cytokinin signaling components and the auxin transport machinery. Furthermore, CRF2, identified as a direct mark of *TARGET OF MONOPTEROS* (TMO3)[43] , might act as an important convergence point to fine-tune the auxin transport activity in response to both auxin and cytokinin. The role of CRFs in the auxin transport regulation is supported by the altered expression of *PIN1* and *PIN7* in *CRF* gain- and loss-of-function lines as well as by the embryonic and root developmental defects in *crf* mutants that are reminiscent of those found in auxin transport mutants[2,11]. By testing the

impact of CRFs on the *PIN* expression in transient protoplast assays, we found that CRF2, CRF3, and CRF6 might act as positive regulators of *PIN* transcription, but *in planta* analyses of *PIN* expression in *crf* mutants and light-dependent *PIN* activation by CRF3 in protoplasts rather suggest that the CRF activities are part of multi-component transcriptional complexes that might depend on conditional and developmental contexts. However, whether CRF3 directly interacts with *PCREs in planta* and/or is part of the *PIN* expression-modulating transcriptional complex remains to be elucidated. Altogether, we propose that, through the transcriptional regulation of *PIN* genes, CRFs mediate cytokinin signals to fine-tune the activity of the polar auxin transport machinery during plant growth and development.

Material and methods

Plant material and growth condition

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *PIN1::PIN1-GFP*[5], *pin7-2*[5], *PIN7::PIN7-GFP*[44], *PIN7::GUS*[2], *RPS5A::CRF2*[43], *35S::EGFP-CRF6* [45]. The previously characterized *CRF* knockout mutants[30] were obtained from various T-DNA insertion mutant seed collections: *crf2-2* and *crf3-1* from the Salk Institute Genomic Analysis Laboratory (SAIL, former GARLIC) T-DNA insertion lines from the Torrey Mesa Research Institute[46] and *crf6-2* from GABI-Kat[47]. Primers and T-DNA accession numbers are listed in Supplemmentary Table 1. Seeds of *Arabidopsis* (accession Columbia-0) were plated and grown on square plates with solid half-strength Murashige and Skoog (MS) medium supplemented with 0.5 g/L MES, 10 g/L Suc, and 0.8% agar. The plates were incubated at 4°C for 48 h to synchronize seed germination and then grown vertically in growth chambers under a 16-h/8-h day/night cycle photoperiod at 18°C.

Cloning and Generation of Transgenic Lines

For promoter analysis of *PIN1* and *PIN7*, particular promoter fragments were amplified by PCR and cloned into the pGEM-T vector. The used primers contained unique restriction sites: *PstI*-sense and *BamHI*-antisense for *PIN1* and *Sal*I-sense and *BamHI*-antisense for *PIN7*, allowing digestion and subsequent cloning into the pGREEN binary vector. The resulting constructs contained transcriptional fusions between the *PIN1* or *PIN7* promoter variants and the enhanced Green Fluorescent Protein (EGFP) with a nuclear localization signal (NLS). Primers used for cloning are listed in Supplementary Table 3 (Chapter3).

The translation fusion Δ*PIN1::PIN1-GFP* was obtained by modifying *PIN1::PIN1*-GFP (in *pBINPLUS* vector backbone[5]) as follows: the *PIN1* promoter sequence from -258 to -2320 relative to the initiating ATG was removed by *Xba*I digestion and replaced by the *PIN1* promoter sequence spanning the -258 to -1212 region. Δ*PIN7::PIN7-GFP* was derived from *PIN7::PIN7-GFP* in *pBINPLUS*[2] by removal of the *Eco*RI fragment. The truncated promoter construct contained 1141 bp upstream from the translational start site.

Expression plasmids were generated by standard molecular biology protocols and Gateway technology (Invitrogen). Open reading frames (ORFs) were amplified from a cDNA template with Pfu DNA Polymerase (Promega) and fused to the Gateway attB sites by PCR. pDONR221 and p4-p1r were used as ENTRY vectors. The structure and sequence of all destination vectors were as described[48,49] and are available online at http://www.psb.ugent.be/gateway/ or otherwise referenced. *35S::CRF3* and *35S::CRF6* were obtained by cloning the ORFs of *CRF3* and of *CRF6* into destination vectors *pK7WG2*.0 and pK7WG2D, respectively. The *CRF3* and *CRF6* (2 kb) promoters were cloned in pMK7S*NFm14GW, generating the *ProCRF3:NLS-GFP-GUS* and *ProCRF6:NLS-GFP-GUS* constructs (transcriptional fusions between the promoters and the EGFP-GUS fusion protein). The *CRF2::CRF2-GFP* protein fusion, used for the ChIP experiments, is a *CRF2* promoter fragment (2 kb upstream from the coding sequence of *CRF2*) cloned into the *PgreenIIK* vector, resulting in a fusion with the NLS and EGFP. All transgenic plants were generated by the floral dip method[50].

Phenotype analysis

For the phenotype analysis of the root parameters (root length and root meristem size) and the lateral root primordia development, 10-20 seedling of 7- and 10-day-old seedlings were processed as described[51]. For the analysis of the root growth kinetics, seedlings were recorded every day for 14 days with an EOS035 Canon Rebel Xti camera. All data were analyzed with with the ImageJ software (NIH; http://rsb.info.nih.gov/ij) as described[14].

Confocal imaging and image analysis

Confocal microscopy images were obtained with the Zeiss LSM 510, Zeiss LSM 710, or Olympus FV10 ASW confocal scanning microscopes. Fluorescence signals were detected for GFP (excitation 488 nm and emission 507 nm) and propidium iodide (excitation 536 nm and emission 617 nm), whereas yellow fluorescent protein (YFP) signals were observed with the

GFP settings. Development of lateral root primordia was followed in real time as described[29]. Fluorescence intensities of the PIN-GFP membrane signals were quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described[52]. The statistical significance was evaluated with the Student's *t*-test.

Histochemical and histological analysis

Samples were stained with GUS and, for microscopy analysis, were cleared as described previously,[51]. All samples were analyzed by differential interference contrast microscopy (Olympus BX51).

Embryos were labeled by immunofluorescence as described[53]. Primary rabbit anti-GFP and rabbit anti-PIN1 antibodies were diluted 1:600 and 1: 400, respectively, and secondary anti-rabbit-Alexa 488 and anti-rabbit Alexa 546 antibodies were diluted 1:600 in 3% bovine serum albumin (BSA) in phosphate buffered saline. Life-imaging was done with a confocal laser scanning microscopy (LSM 510, Zeiss). Images were analyzed wit the LSM Image Browser (Zeiss)

Transient expression in protoplasts

Mesophyll protoplasts were isolated from rosette leaves of 4-week-old *Arabidopsis* plants grown in soil under controlled environmental conditions in a 16-h/8-h light/dark cycle or under continuous light at 21°C. Protoplasts were isolated and transient expression assays were carried out as described[54] with modifications[55]. Protoplasts were co-transfected with 20 μg of a reporter plasmid that contained *fLUC*, a reporter gene driven by the corresponding promoter, 2 μg of normalization plasmid expressing the *Renilla luciferase* (*rLUC*) under the control of the *35S* promoter, and 20 μg effector construct. For the reporter constructs, *pEN-L4-Pro-R1* vector (with Pro representing *PIN1:LUC,* Δ*PIN1::LUC, PIN7::LUC*, and Δ*PIN7::PIN7-GFP*) was recombined together with *pEN-L1-fLUC-L2* by Multisite Gateway LR cloning with *pm42GW7*[49]. For the effector constructs, the *pEN-L1-ORF-R2* plasmids (with ORF representing *CRF2, CRF3*, or *CRF6*) were used to introduce the ORFs by Gateway LR cloning into *p2GW7* for overexpression. The total amount of DNA was equalized in each experiment with the *p2GW7-GUS* mock effector plasmid. After transfection, protoplasts were incubated overnight and then lysed; *fLUC* and *rLUC* activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical errors were corrected by normalization of *fLUC* by the *rLUC* activities. The mean

value was calculated from two measurements and each experiment was repeated at least three times.

Yeast One-Hybrid screen

The yeast strain YM4271 and destination vectors *pDEST-MW1* and *pDEST- MW2* have been described previously[56]. The yeast-one-hybrid screen was done as described in Chapter 3.

ChIP and quantitative PCR

ChIP experiments were done as described[57] with minor modifications. One gram of tissue from 8-day-old plants was harvested and immersed in 1% formaldehyde under vacuum for 10 min. Glycine was added to a final concentration of 0.125 M and incubation was continued for 5 min. After washing, the nuclei were isolated and crosslinked DNA/protein complexes were fragmented by sonication with a Bioruptor sonicator (Diagenode), resulting in fragments of approximately 500 bp. After centrifugation (at 500 *g*), the supernatant was precleared with 80 µl of sheared salmon sperm DNA and protein A agarose (Millipore), of which 10 µl was used as input and the remainder was divided into three samples. To two samples (IP1 and IP2), 25 µl GFP-Trap®_A coupled to agarose beads (Chromotec) was added, whereas to the third sample, which served as IgG control, an equal volume of nonspecific control serum, consisting of sonicated salmon sperm DNA, BSA, and Protein A (Salmon Sperm DNA/Protein-A agarose-50% slurry; Millipore). The samples were incubated overnight and immunoprecipitates were subsequently eluted from the beads. All centrifugation steps with bead-containing samples were done at 500*g*. Proteins were de-crosslinked and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were resuspended in MiliQ water. The concentration of ChIP DNA was measured with the Quant-iT double-stranded DNA HS assay kit (Invitrogen). The SYBR Green I Master kit (Roche Diagnostics) was used for all the quantitative PCRs. *ACTIN2* and promoter regions of *PIN1* (433-512 bp upstream from the start codon) and *PIN7* (357-553 from ATG) were utilized as negative controls. All primers, including those for *PCRE1* and *PCRE7*, are listed in Supplementary Table 1. To analyze the ChIP enrichment from quantitative PCR data, the % input was calculated as: $\%Input=2^{(CtInput-CtChIP)} \times Fd \times 100\%$, with Fd the input dilution factor (1/100). Standard deviations were calculated for IP1 and IP2 as ln(2)*dSD*FC and for IgG as

ln(2)*dSD, with FC the fold change. ChIP data were obtained from single experiments, but similar data were acquired from three independent experiments.

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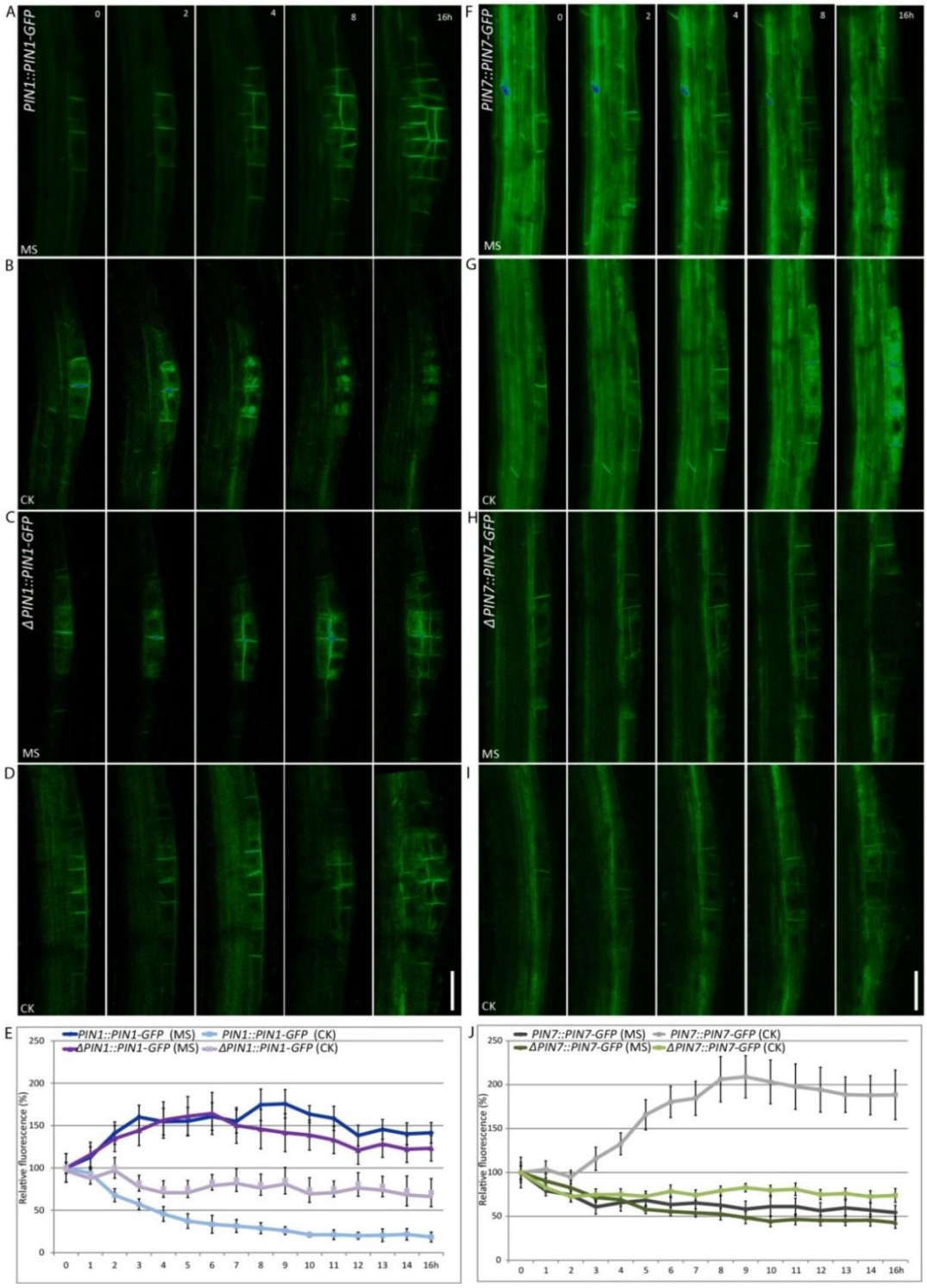
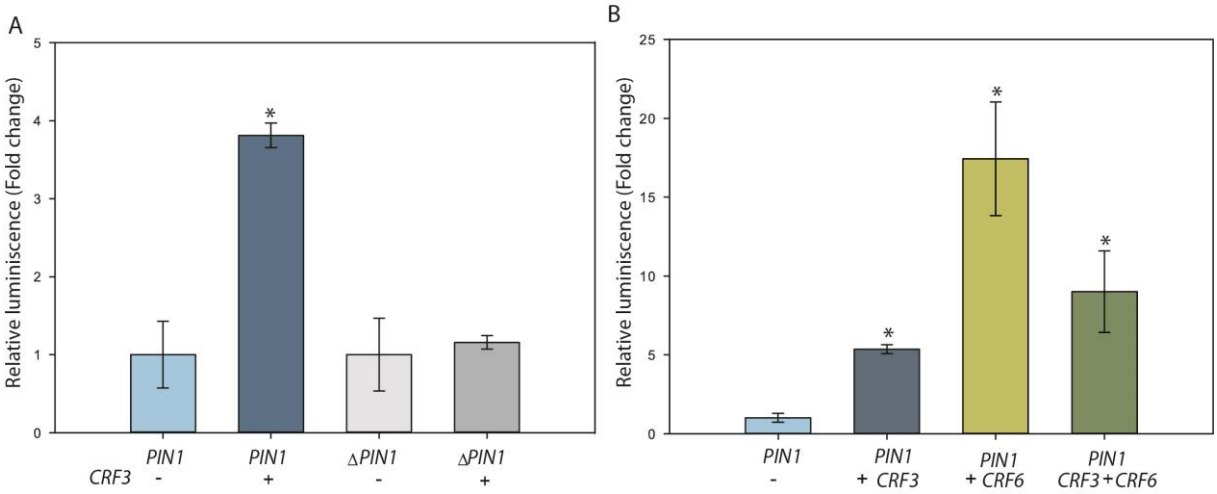
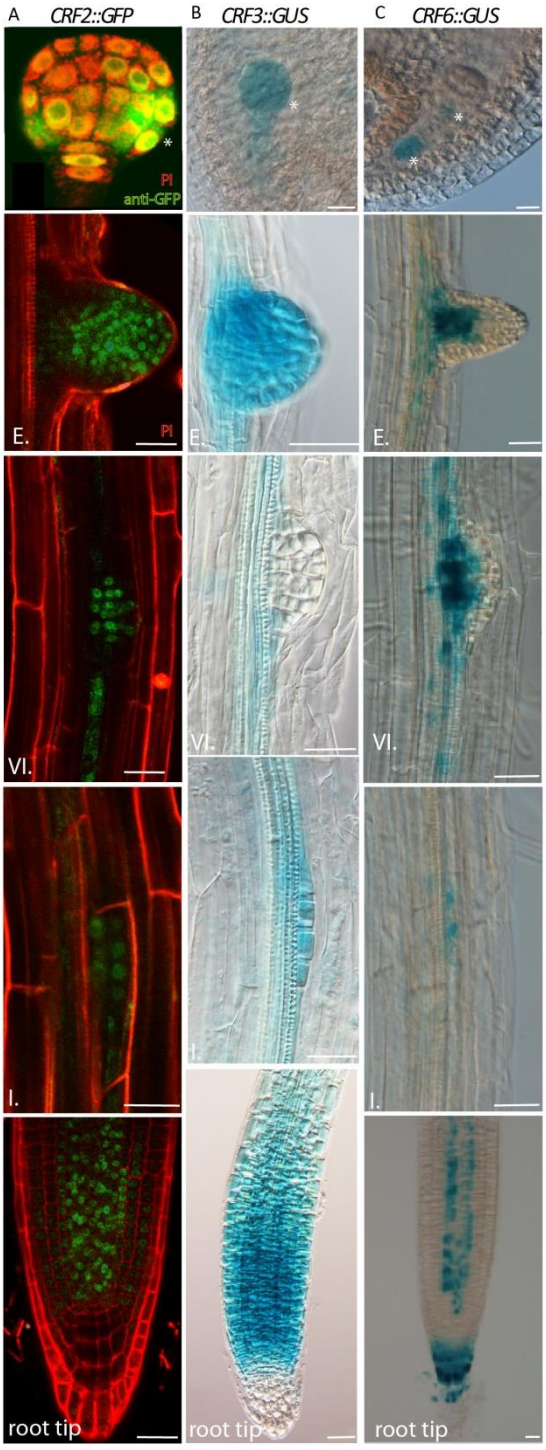
Supplemental data



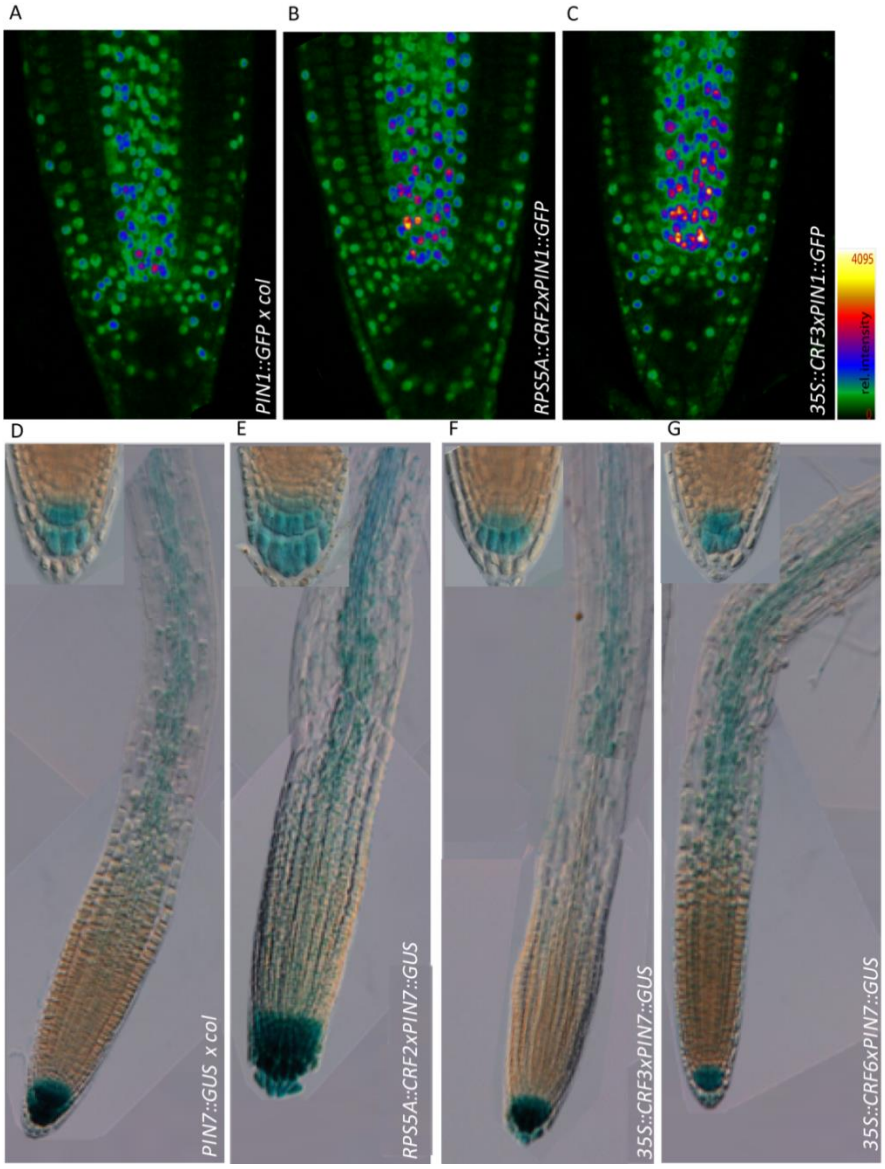
Supplementary Figure 1. ***Truncation of PIN promoter results in cytokinin-insensitive PIN transcription in lateral root primordia***. (A-E) Down-regulation of the *PIN1-GFP* translation fusion expression after cytokinin treatment when driven by full *PIN1* (A, B), but not truncated *ΔPIN1* promoters (C, D). (F-J) Up-regulation of *PIN7-GFP* translation fusion expression after cytokinin treatment when driven by full *PIN7* (F, G), but not truncated *ΔPIN7* promoters (H, J). Quantification of *PIN1-GFP* (E) and PIN7-GFP (I) signals at the anticlinal membranes of stage-I lateral root primordia[51] in1-h intervals after treatment with cytokinin. Seven-day-old roots were treated with control Murashige Skoog medium without or with cytokinin (CK; 2 µM N^6-benzyladenine). Student's *t* test (*p<0.05; n=8-10). Error bars represent standard error. Scale bar 20 µm.



Supplementary Figure 2. CRF3-mediated light-dependent regulation of *PIN1* expression. (A) Significant up-regulation of the *LUCIFERASE* reporter expression when driven by *PIN1*, but not by the truncated *ΔPIN1* promoter, by co-expression of CRF3 in *Arabidopsis* mesophyll protoplasts isolated from plants grown under continuous light at 21°C. (B) Attenuated stimulatory effect of CRF6 on *PIN1::LUC* expression by co-expression of CRF3 with CRF6. Student's *t* test (*p<0.05, n=3).



Supplementary Figure 3. ***Expression of CRF2, CRF3 and CRF6 in early embryos and roots.*** (A) *CRF2::GFP* (B) *CRF3::GUS,* and (C) *CRF6::GUS* expression detected in globular-stage embryos, stage-I and stage-VI lateral root primordia, and emerged (e) root tips. Red staining - propidium iodide. Scale bar 20 µm.



Supplementary Figure 4. ***Altered expression of PIN genes in CRF-overexpressing lines.*** (A-C) Up-regulation of *PIN1::GFP* expression in root meristem provasculature of lines overexpressing *CRF2* (B) and *CRF3* (C). (D-G) Up-regulation of *PIN7::GUS* expression in columella and vasculature of *RPS5A::CRF2* roots (E), reduction in root vasculature of *35S::CRF3* roots (F), and down-regulation in columella, but up-regulation in root vasculature of *35S::CRF6*-overexpressing lines A semi-quantitative color-coded heat-map of the GFP fluorescence intensity is provided. Analysis of 7-day-old (A-C) and 5-day-old (D-G) roots (n=10-15 seedlings).

Supplementary Table1. List of primers.

Name	Sequence (5'->3')
Clonning primers	
ΔPIN1_XbaI_Fw	CTCTAGAGTAAGCAAACCAGAAC
ΔPIN1_XbaI_Rv	CTCTAGATATTATATTCAAAAGTAGTTTAC
attB4_PIN1_Fw	GGGGACAACTTTGTATAGAAAAGTTGCTTCTAGAGTCGACTTTTAG
attB1_PIN1_RV	GGGGACTGCTTTTTTGTACAAACTTGCCTTTTGTTCGCCGGAGAA
attB4_ΔPIN1_Fw	GGGGACAACTTTGTATAGAAAAGTTGCTGTAAGCAAACCAGAACCA
attB1_ΔPIN1_RV	GGGGACTGCTTTTTTGTACAAACTTGCCTTTTGTTCGCCGGAGAA
attB4_PIN7_Fw	GGGGACAACTTTGTATAGAAAAGTTGCTGAAGAATGCGAAGAAGAG
attB1_PIN7_RV	GGGGACTGCTTTTTTGTACAAACTTGCTGTTGTTCGCCGGAGTGG
attB4_ΔPIN7_Fw	GGGGACAACTTTGTATAGAAAAGTTGCTGAAGAATGCGAAGAAGAG
attB1_ΔPIN7_RV	GGGGACTGCTTTTTTGTACAAACTTGCTTCTTGGGCCACTTAACG
attB1_CRF2-ORF_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTA ATGGAAGCGGAGAAGAAA
attB2_CRF2-ORF_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTA TTAAACAGCTAAAAGAGGAT
attB1_CRF3-ORF_Fw	GGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGACGAATATATTGAT
attB2_CRF3-ORF_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTATAAGCAACTAATAGATCT
attB1_CRF6-ORF_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGAGACGAACGAGA
attB2_CRF6-ORF_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAATCGAAAGAGTGATG
qPCR-ChIP primers	
PCRE1_ChIP_Fw	GTTGAAAACATTGGAAAAATAAAAAG
PCRE1_ChIP_RV	TCAGTCTGATGTGTTCTTTAATACT
P1[-512-433]_Fw	TTTGGACTCCGCAAAGCGCA
P1[-512-433]_RV	AAAAATGGTGGTTGATGGGTGCGTGG
PCRE7_ChIP_Fw	GTTTTGGACCGGCATTTACG
PCRE7_ChIP_RV	TGAGATTTTTTAGAGTGGACTGACTG
P7[-533-357]_Fw	GTTTTGGACCGGCATTTACGTG
P1[-533-357]_RV	GTGGACTGACTGTCTACATGTCAC
Actin_ChIP_Fw	TATCGTTGGCCGTCCACGTC
Actin_ChIP_RV	ACGTTTCGACTGTGCCTCGT

Supplementary Table1. List of primers for genotyping.

Locus	mutant	T-DNA name	LP	RP
AT4G23750	*crf2-2*	SAIL_371_D04	TGGAAGCGGAGAAGAAAATGG	TAAAAGAGGATCCGACCCGAA
AT5G53290	*crf3-1*	Garlic_240_H09.b.1a	CAAAATCACTAAACGTTGAACCCA	CCGACCATTGAAGTACACAGAG
AT3G61630	*crf6-2*	gabi_541G11	TCGAAGAAATGATCCACATCC	GGTGAATCTTCTTCCTCCACC

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Chapter 4.

Conclusions and Perspectives

Conclusions and Perspectives

Since, several decades ago, Skoog and Miller demonstrated the antagonistic behavior of auxin and cytokinin (CK) in promoting the growth of roots and shoots[1], an enormous progress has been made in understanding the regulation of the stem cell niche and cell fate in both shoot and root apical meristems. Developmental processes, such as the maintenance of root meristems[2], lateral root formation[3], shoot branching[4], and *de novo* auxin-induced organogenesis[5], are fine-tuned by the crosstalk between these two hormones. Auxin inhibits CK biosynthesis and signaling[6–8], whereas CK antagonistically affects the auxin transport, distribution and signaling[2,9–13]. In particular, the regulation of auxin transport and distribution plays an important role in many of the above mentioned aspect of plant development. We showed that CK modulates expression of PIN-FORMED (PIN) auxin efflux carriers, which are determinants of the polar auxin transport, at the transcriptional level to control the root meristem size[12] (Chapter 2). In line with our observations, another study demonstrated that CK affects root meristem function: CK signaling via the ARABIDOPSIS HISTIDINE KINASE3 (AHK3)-ARABIDOPSIS RESPONSE REGULATOR (ARR1), ARR12 module induces the negative regulator of auxin responses SHORT HYPOCOTYL2/INDOLE-3-ACETIC ACID3[2], subsequently leading to the down-regulation of PIN1 transcription. However, specific components mediating a direct interaction between CK signaling and *PIN* expression are still unknown.

In this thesis, our aim was to identify the direct regulators of the *PIN* expression in response to CK signaling. First, we analyzed the promoter sequences of *PIN1* and *PIN7* for the presence of CK-responsive elements. To this end, we designed promoter deletion constructs and observed the insensitivity to CK treatments upon removal of a certain 200-bp region from the *PIN1* or *PIN7* promoters. This reduced response to CK was also reflected in CK-mediated aspects of plant development in transgenic plants in which the *PIN* expression was driven by truncated promoters without specific CK-responsive elements, designated *PIN* CK response elements or *PCRE*s) (Chapters 3 and 4).

Next, we attempted to elucidate the transcriptional network that controls the *PIN* expression through direct binding to the PCREs. Therefore, the identified *PCRE*s were systematically screened for potential interactors by the Yeast-one-hybrid (Y1H) assay (Chapter 3), a method that allows the identification of protein–DNA interactions on a large-scale[14].

To detect such interactions, libraries prepared from total mRNA might not be appropriate because transcription factor (TF) genes are frequently expressed at low levels. Moreover, proteins with high affinity to unspecific DNA regions may frequently lead to false positives and the identification of low-abundant TFs from such libraries would require the screening of a large number (millions) of colonies. Recently, several research groups generated normalized collections consisting of *Arabidopsis thaliana* TFs only[15–17]. TF collections are arrayed in 96-well plates that can can be transferred independently in a high-throughput mating-based setup[16,17] or by haploid transformations (Chapter 3), providing a direct way to identify TF-DNA interactions. In this manner, each cDNA clone of the collection is surely tested for interaction with the bait DNA. Compared to plant promoters, yeast promoters are rather short. Therefore, *cis* elements more than 1000 bp away from the transcription initiation cannot activate expression, even after binding with a TF. Moreover, transcription initiated proximally to the upstream activation sequence (UAS), which is located more than 300 bp upstream of the reporter sequence, competes with the UAS of the reporter gene located downstream[18]. This drawback restricts the use of the Y1H systems to short promoter fragments or multimerized *cis* elements. The recently developed protoplast transactivation (PTA) system can also be used with an arrayed TF collection (PTA)[19] and has the potential to overcome this limitation. Furthermore, protoplasts provide the plant-specific cofactors that could be crucial for certain interactions and are not present in yeast cells.

In the presented Y1H screen, we could detect a number of candidate interactors, of which many belong to large plant TF families, hinting at a possible gene redundancy, possibly leading to weak phenotypes of single loss-of-function lines, as observed, for instance, in the case of CK signaling and biosynthesis mutants[20–24]. Therefore, the generation of multiple mutants would be necessary. However, single knockout mutants of several selected TFs displayed an impaired phenotype in CK-regulated processes, such as root growth and lateral root development, and could be divided in positive and negative regulators of the root development (Chapter 3). Nevertheless, a more detailed analysis of their ability to induce or inhibit *PIN* transcription would give an indication about their involvement in transcriptional complexes that control auxin transport and distribution in response to CK.

For the CK response factors (CRFs), a subset of TFs belonging to a small subclass of the APETALA2/ETHYLENE RESPONSE FACTOR family that are induced by CK[25], we could confirm independently that they bind to *PCREs* in the *PIN* promoters and affect the promoter activity and *PIN* expression. Moreover, CRF loss- and gain-of-function mutants exhibited

defects in embryogenesis, cotyledons, and root and lateral root development that resembled the phenotype of auxin distribution mutants, further strengthening their role as integrative factors in the auxin-CK crosstalk during plant development (Chapter 4). In addition, CRF2 could represent an additional level of integration because it is a transcriptional target of MONOPTEROS (MP/AUXIN REPONSE FACTOR5), an auxin signaling component[26], and thus might interpret both auxin and cytokinin signals into developmental changes (Figure 1).

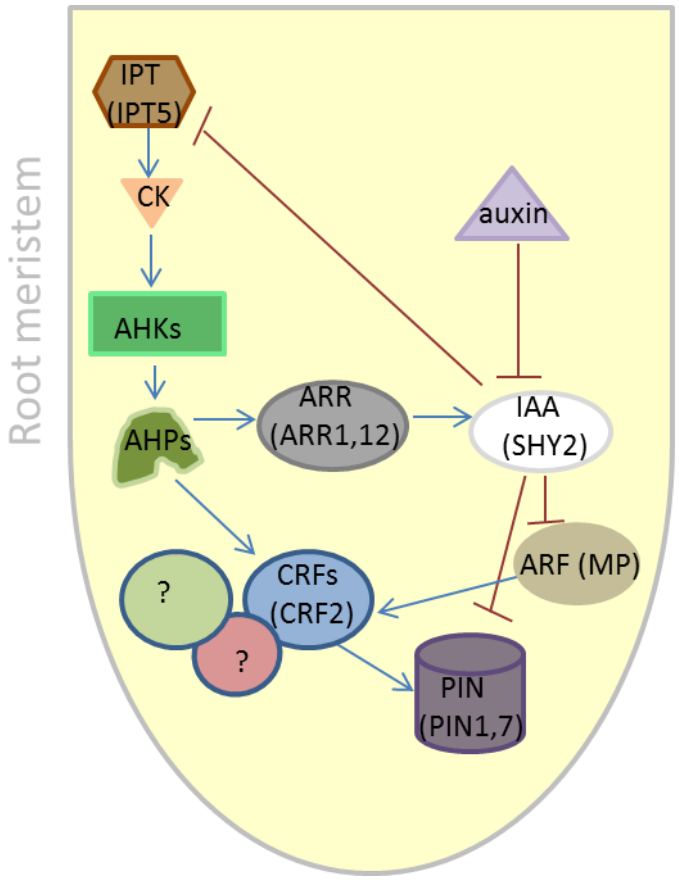

Figure 1. Model of CK-dependent regulation of *PIN* expression. During root meristem development, CK induces via AHK3-ARR1,ARR12 signaling a negative regulator of auxin response, to SHY2/IAA3, which, in turn, inhibits the CK biosynthesis gene encoding ISOPENTENYL TRANSFERASE5 (*IPT5*)[2]. In addition to this SHY2-mediated auxin-CK crosstalk, CRFs bind specifically to *PIN1* and *PIN7* promoters and control root development in response to the CK signal. CRF2, a direct target of MONOPTEROS (MP)[26], might regulate the auxin transport activity in response to both auxin and CK and, most probably, act as a component in a multi-subunit transcriptional complex.

CRFs contain the AP2 DNA-binding domain that is capable to interact with slightly different DNA sequences while conserving a strong affinity for G/C-rich motifs[27–29]. CRFs are further distinct from other members of the ERF subfamily by the presence of a B-5 domain[30], of which the function is still unknown. When we take into account another example

of RELATED TO ABSCISIC ACID-INSENSITIVE3/VIVIPAROUS1 (RAV) proteins that, besides an AP2 domain, contains a B-3 domain, which acts as a DNA-binding domain that recognizes different DNA-binding motifs, and the fact that we could not confirm the binding of CRF to the GCC box in the *PIN1* promoter by the ChIP analysis, we can hypothesize that the binding specificity of CRFS might slightly differ from the canonical GCC box sequence.

The candidate binding site might be obtained by motif prediction based on the *PCRE1* and *PCRE7* alignments or by identification of a sequence motif that is conserved within the targert gene promoters, which could be selected from microarray analysis of *CRF*-overexpressing transgenic plants. The microarray approach is built on the idea that target gene sets are coordinately up-regulated in response to the high expression of selected TFs and that this control is achieved through the interaction of those TFs with conserved *cis*-regulatory motifs in the target gene promoters[31].

In our study, the computational analysis of the identified CK response elements (*PCRE1* and *PCRE7*) provided us with a candidate motif of 12 bp (AAGCAATTGCTT) that occurs twice in each element (Chapter 3) and, interestingly, contains two GC repeats. Considering the strong affinity of the AP2 domain to G/C-rich motifs, this sequence feature might be improtant for the specific binding of CRF TFs. The aim of our future work is to test the functionality of the identified motif in transient expression assays. In addition, confirmation of the binding to this motif by some of the other retrieved TFs or to additional motifs found by searches in databases and the literature could uncover new PIN transcription regulators.

Gel mobility shift assay[32]or DNAseI footprinting[33] could be used to prove that certain TFs bind to DNA fragments that contain predicted DNA-binding sites. Such experimental approaches are restricted by the need to purify TF protein beforehand and will only reflect *in vitro* binding activities. Alternatively, additional Y1H screenings with multiple copies of the predicted binding sites could determine protein-DNA interactions through the transcriptional activation of reporter genes *in vivo*. Whereas these assays are effective for the analysis of simple protein-DNA interactions, the absence of any plant-derived factors other than the TFs under investigation can restrict the applicability of this technique. To overcome this limitation, the more recent chromatin immunoprecipitation (ChIP) techniques have been used to immunoprecipitate DNA associated with a TF of interest in plants[34]. Thus, the combination of bioinformatics approaches supported by experimental data will allow us to predict and formulate new interactions that regulate various aspects of plant development.

In this thesis, we uncovered a missing link between the CK-dependent control of *PIN* transcription and plant-specific developmental processes by identifying CRF TFs as binders to specific PIN promoter elements, but they are clearly not the only players in this control. Different CRF family members exhibit specific functions that depend on the tissue and specific conditions[26,35,36] (Chapter 4), most probably through their interaction with each other[37] and/or with other components of transcriptional complexes (Figure 1).

In this light, the main challenge in the future will be to unravel the diversity and flexibility of the different CRF TFs as well as to identify their potential binding partners that act in the same auxin-CK crosstalk-mediating transcriptional complex. We showed that CRFs play important roles in embryogenesis and root development (Chapter 4). In addition, the high levels of *CRF1*, *CRF2*, and *CRF6* expression seen in the meristematic tips of shoot apices[38] suggest that these genes are involved in shoot apical meristem maintenance as well, a process known to be mediated by the auxin-CK crosstalk. Therefore, it would be interesting to assess the function of CRF family members during shoot apical meristem formation. However, the CRF-independent regulation of the PIN activity via other TFs isolated in the Y1H screen cannot be excluded. Hence, the characterization of the functionality and biological importance of these TFs could provide new insights into the auxin-CK interplay. With the wide range of genetic and molecular tools available for the *Arabidopsis* research, the exact mechanism should be soon revealed.

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Summary

Summary

In recent years, the research has provided a better comprehension of the extensive crosstalk between auxin and cytokinin (and various other hormones). Antagonistic interactions that have been revealed over 50 years ago[1] are now being understood at the genetic and molecular levels.

The plant root is widely used as a model to investigate hormonal crosstalk in a wider context. The key regulators of root growth and meristem maintenance are auxin, cytokinin (CK) and ethylene[2–5]. CK has been shown to regulate the biosynthesis of ethylene[6] that, in turn, affects CK signaling[7]. In addition, ethylene also induces auxin responses in the root meristem elongation zone[8–10]. In the first chapter, we demonstrated that the auxin-CK interaction is involved in the control of the root meristem size. This control is independent of ethylene and is mediated through the CK-dependent *PIN-FORMED* (PIN) gene transcription regulation. Our research contributes to the understanding of the crosstalk between these two hormones on the transcriptional level, of which the importance has been highlighted in several independent studies[3,11,12].

To gain more information on this regulation, we investigated *PIN1* and *PIN7* promoter regions for *cis*-regulatory elements and identified the *PIN CYTOKININ RESPONSE ELEMENT* (*PCRE*) in both promoters, of which the removal leads to CK-insensitive *PIN* transcription (Chapter 3). In addition, uncoupling of the *PIN* expression from the CK control affects root growth and lateral root development, underlining the key role of cytokinin dependent regulation of the *PIN* expression for normal plant growth and development (Chapter 4).

Next, we aimed to unravel the transcriptional network that mediates this control. By means of the yeast-one-hybrid (Y1H) system and through the phenotypic analysis of loss-of-function mutants, we could identify several candidates that act as regulators of the primary and lateral root growth (Chapters 3 and 4).

Interaction of the CYTOKININ RESPONSE REGULATORS (CRFs) with the *PCRE*s could be confirmed *in vivo* as well as their impact on *PIN* expression and plant development from early embryogenesis (Chapter 4). We proposed that the CRFs are the main players in the transcriptional network that controls *PIN* expression in response to CK signals. Additional

components might act as regulators in this network and it remains to be elucidated whether some of the other TFs retrieved in the Y1H assay TFs are part of this system.

Taken together, our results indicate that an approach that includes identification of DNA-binding motifs in selected promoter regions, screening of expression databases for expression pattern of newly found TFs, followed by in planta confirmation of interactions by protoplast transient expression assays or the chromatin immunoprecipitation technique, and the phenotypic analysis of mutants of the identified genes may be more restrictive and specific to detect relevant protein-DNA interactions. The combination of in vitro studies and in vivo functional analysis of selected interactions will contribute to the understanding of gene regulation in the auxin-CK hormonal crosstalk context.

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Curriculum vitae

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Šimášková, M., Obrien, J.A., Van Noorden, G., Vieten, A., De Clercq, I., Van Haperen, A., Cuesta, C., Hoyerova, K., Marhavy, P., Van Breusegem, F., Murphy, A., Friml, J., Weijers, D., Beeckman, T., and E. Benková. The cytokinin response factors CRFs control *PIN-like* gene transcription (manuscript in preparation)

Poster presentation

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